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On the Extra-Ovarian Origin of Estrogen

By

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INTRODUCTION

The main source of estrogen is thought to be ovaries and adrenal glands in a female, but estrogen can be found from urine after combined oophorectomy-adrenalectomy.[1),2)] The source of estrogen excreted after oophorectomy-adrena lectomy is not clear. I already explained "on extra-sexual estrogen" with co-workers.[3)] It has been reported that the sources of estrogens after oophorectomy-adrenalectomy in some animals seem to be accessory adrenal tissue, but all of operated animals have not always the accessory adrenal tissue.[4),5)] At present, estrogen secretion from accessory adrenal tissue is inferred from the histological evidence, and the relation between secretion or excretion of estrogen and organs is not explained even now.

Although a considerable number of publication have appeared in recent years, little is known about the relationship of the organs and the estrogen secretion, so I have done the investigation on estrogens of extra-sexual glands.

MATERIAL AND METHOD

1) Experimental Materials

a) I inquired into 10 oophorectomized women from 42 to 51 years of age, who were patients of uterus cancer. 24 hour urine was collected before operation, and estrogens were extracted. I collected urines of them 2, 7, 12, 17, 22, 27 and 32 days after operation, but 5 women's urines were collected 60 days later and the experiment was made.

b) The animals used in these experiments, five female rabbits weighing 2 to 3 kg, were placed in individual cages of double wire-mesh floor, and 24 hours urine was collected. Each of adrenalectomized rabbits was dosed 2.5 mg DOCA daily.

c) The bled rabbits were those other than the rabbits used in the collection of

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urine, blood was drawn 10 ml directly from the hearts of the rabbits shortly before and 25 days after oophorectomy. The serum was separated and stored at 0°C until analysis.

d) 25 g of tissues were used to estimate the estrogen in rabbit organ tissues. Oophorectomized rabbits were killed 25 days after the operation and tissues were minced and mixed with 80 % (v/v) ethanol by 30 min. after extirpation.

e) In order to investigate the conversion to estrogen from another steroid in each organ tissue, I examined the conversion of testosterone to estrogen with rabbit uterus and kidney tissues.

2) Experimental Methods

Fresh tissues of adult female rabbit organs were brought frozen to the laboratory. 20 ml of Krebs-Ringer phosphate solution (pH. 7.4) and 2 g of the

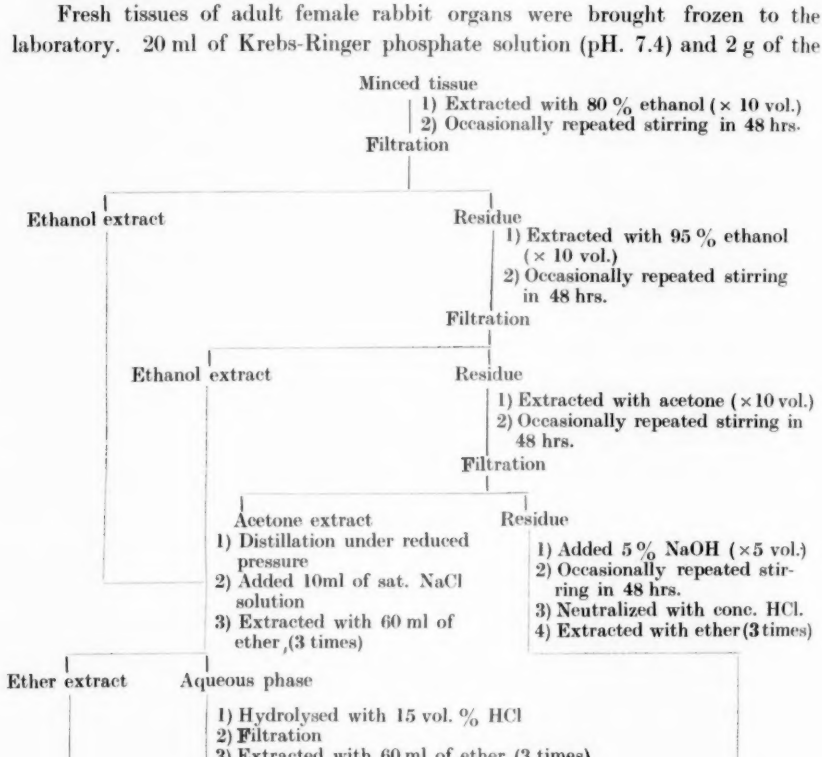
Minced tissue
1) Extracted with 80 % ethanol (× 10 vol.)
2) Occasionally repeated stirring in 48 hrs.
Filtration
Ethanol extract
Residue
1) Extracted with 95 % ethanol (× 10 vol.)
2) Occasionally repeated stirring in 48 hrs.
Filtration
Ethanol extract
Residue
1) Extracted with acetone (×10 vol.)
2) Occasionally repeated stirring in 48 hrs.
Filtration
Acetone extract
1) Distillation under reduced pressure
2) Added 10ml of sat. NaCl solution
3) Extracted with 60 ml of ether (3 times)
Residue
1) Added 5 % NaOH (×5 vol.)
2) Occasionally repeated stirring in 48 hrs.
3) Neutralized with conc. HCl.
4) Extracted with ether (3 times)
Ether extract
Aqueous phase
1) Hydrolysed with 15 vol. % HCl
2) Filtration
3) Extracted with 60 ml of ether (3 times)
Ether extract
Aqueous phase, discarded
"Free" estrogen fraction
(1)
"Conjugated" estrogen fraction
(2)
"Protein-bound" estrogen fraction
(3)

Fig. 1. Estimation of estrogen in tissue.

sliced tissues were put in a Warburg bottle, and shaked.

A dosis of precursor was added and stirred at the speed of 60 rotations/min. at 37°C for 3 hours. The identification and determination of blood estrogen were put in practice by the modified method which has been described elsewhere.[6),7)] Urinary estrogen was determined chemically by the method previously described.[6),7)] Extracting Estrogen from the organ tissues was effected by the modified Mitchell & Davies' method (Figs. 1 and 2). Extraction of estrogen from the incubated tissue was made by the method of extracting "free estrogen" in Figs. 1 and 2.

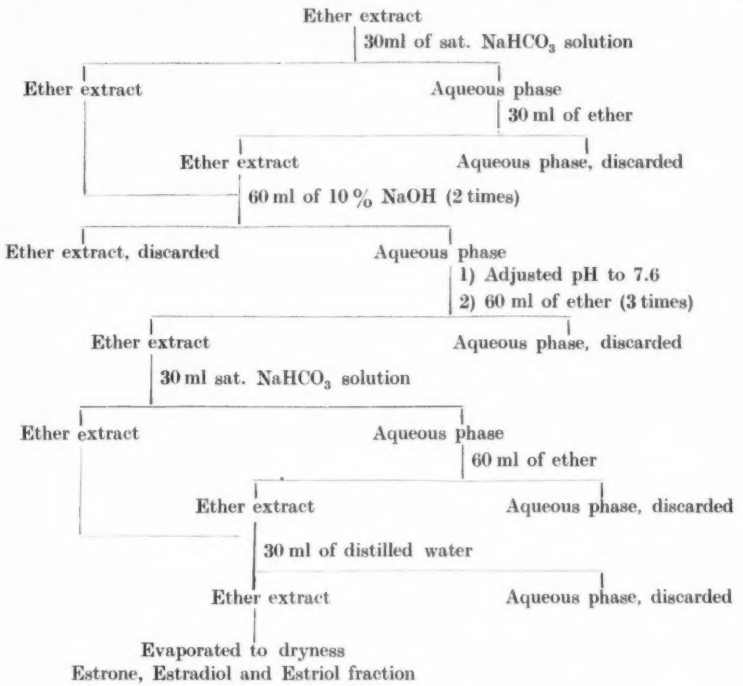

Fig. 2. Fractions 1, 2 and 3 were then treated as follows.

The fraction of extracted estrogen was made by the method of Suzuki & Mori.[9)] For purifying and fractionating the estrogen extracted from incubated tissue, it was first subjected to alumina column chromatography and then to paper chromatography according to Axerlord's method in principle. The details of the method have been given in a previous report[11)]. The content of estrogen was determined by fluorescence photometry.

EXPERIMENTAL RESULTS

1) Urinary estrogen excretion in climacteric women after oophorectomy

Figs. 3 and 4 set forth diagrammatically the procedure. The average values of urinary estrogen excretion of 10 climacteric women were 39.22±20.17 μg. and after oophorectomy it decreased drastically to 29.30±15.23 μg, but after that increased gradually, 12 days after the operation it came back to the approximate value of the previous level. After that, the estrogen excretion decreased slowly, 32 days after the operation it reduced to 17.85±6.63 μg, less than a half of the pre-operation level. 60 days later, the average values of urinary estrogen excretion were 13.2 μg in 5 women. Of the estrogen fraction, estrone was excreted 19.66 ±11.36 μg before oophorectomy, but reduced to 14.15±6.85 μg after the operation, after that estrone excretion in the urine increased gradually, 22 days later 21.95±8.78 μg of estrone could be detected in the urine, and these amounts were larger than at the pre-operation level. However, estrone excretion decreased slowly again, and 32 days after the operation it was excreted 9.00±3.05 μg, less than a half of the previous level. The values of 60 days later were approximately the same as those of 32 days later.

Estradiol fraction was detected 11.08±5.20 μg before the operation, and after oophorectomy it decreased markedly at one time. Urinary estradiol excretion 17 days after the operation increased to 8.58±4.07 μg, but after that decreased again, and the amount excreted was 3.49±1.69 μg, less than one thirds of the pre-operation value. The decrease rate of estradiol fraction was most distinct among the three fractions. The urinary estradiol fraction of 60 days was reduced gradually and the amount was 2.25 μg, but a small amount of estradiol was found in all samples.

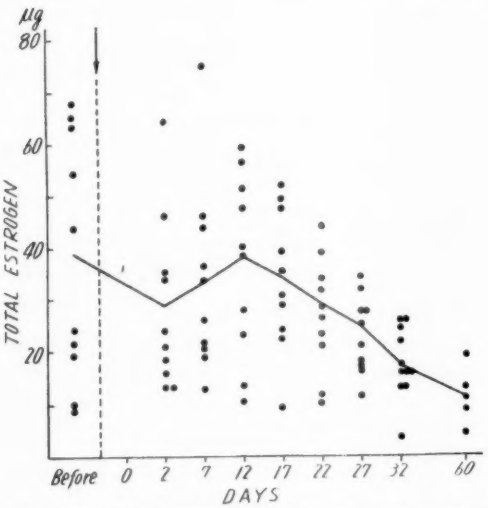

Fig. 3. Urinary estrogen excretion before and after oophorectomy of climacteric women. (Total estrogen : μ/24 hours.)

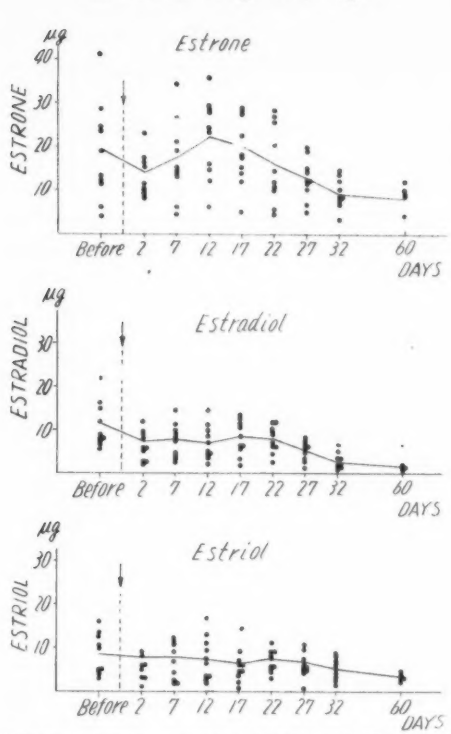

Fig. 4. Urinary estrogen excretion before and after oophorectomy of climacteric women. (Fraction : μg/24 hours.)

Estriol fraction was 8.44±4.32 μg before oophorectomy, but after the operation it decreased slowly, 32 days later the amount being 5.36±2.10 μg and the values coincided with two thirds of the pre-operation level. The estriol excretion of 60 days later was approximately equal to the value of 32 days later.

2) Urinary estrogen excretion before and after oophorectomy-adrenalectomy of rabbits

Figs. 5, and 6 are diagrams to illustrate this relation. Before oophorectomy, 2.49±0.63 μg of total estrogen was found in the urine, and after the operation estrogen excretion decreased at one time. After that, estrogen level was enhanced gradually, 16 days later were excreted 2.26±1.31 μg of almost the same as the pre-operation level, but there were some individual differences. Then, the excretions were lowered gradually, 28 days later were found 1.81±0.82 μg of estrogen and the values were similar to those of the pre-operation level.

After adrenalectomy besides oophorectomy, the total estrogen was reduced rapidly, but could be detected 0.43 μg 12 days after the adrenalectomy.

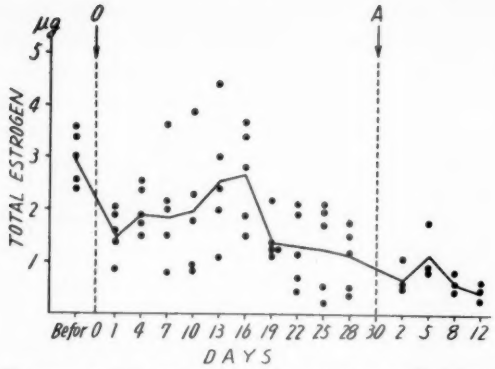

Fig. 5. Urinary estrogen excretion before and after oophorectomy-adrenalectomy of rabbits. (Total estrogen : μg/24 hours)
O : Oophorectomy, A : Adrenalectomy

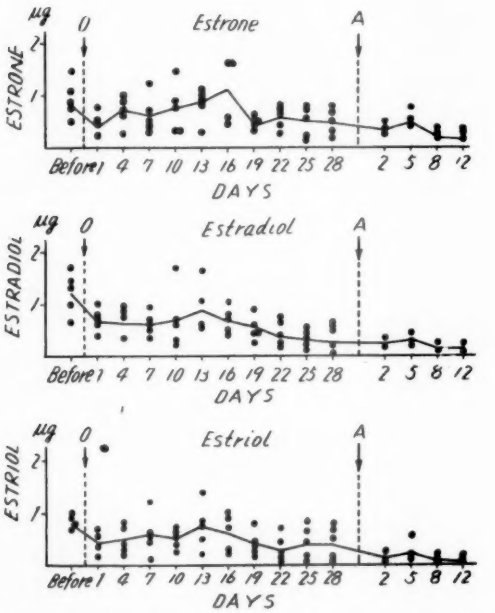

Fig. 6. Urinary estrogen excretion before and after oophorectomy-adrenalectomy of rabbits. (Fraction : μg/24 hours)
O : Oophorectomy, A : Adrenalectomy

On the survey of the estrogen fractions after oophorectomy, estradiol was greatest, the second was estrone, then estriol in quantity, but the difference was not significant.

After oophorectomy, estrone fraction decreased markedly, after that, at one time increased gradually, 16 days later were excreted $1.36\pm0.85\,\mu g$, more than the pre-operation level. But there were some individual variations. 28 days after the operation estrone fraction was found $0.47\pm0.24\,\mu g$ in the urine.

After adrenalectomy, estrone decreased drastically, 12 days later were excreted $0.12\,\mu g$ on the average, less than a half of the pre-operation level in the urine, but a small amount of estrone could be detected in all specimens.

Estradiol fraction was reduced rapidly after oophorectomy, then increased at the one time and 13 days later were excreted $0.96\pm0.39\,\mu g$. Then, estradiol was reduced gradually again, 28 days later were excreted almost the same as one thirds of the previous level and the decreased more and more after adrenalectomy, the mean value of $0.15\,\mu g$ was found in the urine 12 days after adrenalectomy.

Estriol fraction decreased markedly after oophorectomy, 12 days later it increased to $0.74\pm0.40\,\mu g$ at one time, but then decreased again. 28 days after oophorectomy, $0.42\pm0.33\,\mu g$ of estriol were found in the urine. After adrenalectomy, estriol fraction was reduced apparently, but 12 days later $0.08\,\mu g$ of estriol could be detected in the urine.

3) Blood estrogen before and after oophorectomy of adult female rabbits

Table I shows blood estrogen value per serum 10 ml of untreated adult female rabbits. The blood estrogens of 5 untreated rabbits were $2.33\pm0.24\,\mu g$.

TABLE I. Blood Estrogen of Adult Female Rabbits. (μg per serum 10 ml)

	Free estrogen			Protein bound estrogen			
Sample no.	Estrone	Estradiol	Estriol	Estrone	Estradiol	Estriol	Total estrogen
1	0.70	0.23	0.17	0.51	0.22	0.22	2.05
2	0.94	0.18	0.21	0.85	0.38	0.15	2.71
3	0.37	0.24	0.18	0.82	0.30	0.21	2.12
4	1.01	0.34	0.21	0.41	0.17	0.24	2.38
5	0.82	0.27	0.27	0.68	0.31	0.21	2.48
Mean	0.77 ± 0.22	0.25 ± 0.05	0.21 ± 0.03	0.65 ± 0.17	0.28 ± 0.07	0.21 ± 0.03	2.35 ± 0.24

25 days after oophorectomy, blood estrogen decreased markedly, but could be detected $1.31\,\mu g$ on an average of 4 specimens (Table II). Of the estrogen fraction, each fraction in free and protein bound estrogen was reduced together. Especially, estrone and estradiol decreased extremely, but on the rate of estriol/estradiol+estrone any difference between free and protein bound estrogens could not be found.

TABLE II. Blood Estrogen of Oophorectomized Adult Rabbits.
(μg per serum 10 ml)

	Free estrogen			Protein bound estrogen			
Sample no.	Estrone	Estradiol	Estriol	Estrone	Estradiol	Estriol	Total estrogen
1	0.51	0.17	0.27	0.23	0.22	0.27	1.67
2	0.36	0.22	0.17	0.26	0.24	0.12	1.37
3	0.22	0.12	0.11	0.35	0.14	0.12	1.06
4	0.72	0.25	0.21	0.52	0.24	0.20	2.14
Mean	0.45	0.19	0.19	0.34	0.21	0.18	1.56

4) Estrogen in organ tissues of adult female rabbits

With two procedures, I proved estrogen in each organ tissues of rabbits. One was extracted by the direct method, the other by incubation with Krebs Ringer phosphate. The estrogen extracted directly from 25 g of each organ was found in a comparatively large quantity in brain, lung, kidney, uterus, fat and muscle. The quantity of estrogen extracted after incubation was largest in fat and brain, the following in liver, uterus, lung, kidney, muscle and bowel (TABLES III, and IV).

On the investigation of estrogen fraction, both in direct and in post-incubative extraction, estrone and estradiol were proved in a large quantity in all tissues, and a comparatively great deal of estriol in fat and liver also. In comparison with the estrogen extracted after incubation and the directly extracted one, one was proved from 2 to 7 times of the others.

5) Alteration of quantity of estrogen in rabbit organ tissues after oophorectomy

Organ tissues, in which a comparatively great deal of estrogen was proved according to the incubation experiment, were examined in 4 rabbits on their quantity of estrogen after oophorectomy. The extraction of estrogen was made without incubation by the method described in Figs. 1 and 2.

a) Fat: The estrogen contained in fat (100 g) of 5 untreated rabbits can be seen in TABLE V. Free estrogen showed a mean value of 15.78 ± 5.72 μg, conjugated estrogen 6.94 ± 0.35 μg, protein bound estrogen 8.74 ± 1.34 μg and free estrogen could be detected with the greatest quantity in fat. Of the estrogen fraction, estrone and estradiol were found plentifully and they took 80% in the total estrogen. Especially, estrone could be detected in a great quantity in free and protein bound estrogen; it took about a half of total estrogen.

The greater part of the estrogen contained in 100 g of rabbit fat of 25 days after oophorectomy was free estrogen and were extracted 10.73 ± 3.76 μg on an average (TABLE VI). After the operation free estrogen decreased apparently in comparison with the pre-operation level, there was a significant difference, but conjugated and protein bound estrogen were scarcely changed in quantity.

TABLE III. Estrogen in Rabbit Tissues. (I) (μg/100 g)

Material	Case		Estrone	Estradiol	Estriol
Brain	2	Free	7.78	6.42	4.05
		Conjugated	4.42	5.47	2.94
		Protein bound	5.36	4.94	3.47
Lung	3	Free	6.64	1.70	0.94
		Conjugated	1.64	0.60	0.58
		Protein bound	4.44	1.80	1.90
Liver	2	Free	1.86	1.16	2.10
		Conjugated	0.46	0.46	0.48
		Protein bound	2.56	1.20	0.82
Kidney	5	Free	8.19	5.01	5.72
		Conjugated	5.35	2.60	2.23
		Protein bound	5.46	2.03	2.83
Bowel	2	Free	0.90	0.42	0.20
		Conjugated	0.86	0.40	0.30
		Protein bound	6.10	2.82	1.68
Uterus	5	Free	9.16	5.26	4.76
		Conjugated	5.01	3.35	1.52
		Protein bound	3.77	2.49	2.57
Muscle	5	Free	7.66	4.71	3.50
		Conjugated	3.22	1.61	1.90
		Protein bound	3.98	2.22	2.92
Fat	5	Free	6.20	5.50	4.06
		Conjugated	3.26	2.26	1.41
		Protein bound	4.42	2.78	1.58

TABLE IV. Estrogen in Rabbit Tissues. (II) (μg per 2g)

Material	Case	Estrone	Estradiol	Estriol	Total estrogen
Brain	5	0.96	0.38	0.30	1.64
Lung	5	0.73	0.35	0.24	1.32
Liver	5	0.74	0.31	0.37	1.42
Kidney	5	0.69	0.33	0.21	1.23
Bowel	5	0.69	0.27	0.19	1.15
Uterus	5	0.66	0.40	0.31	1.37
Muscle	5	0.67	0.27	0.24	1.18
Fat	5	0.80	1.14	0.61	2.25

(Incubated at 37°C for 3 hrs. with Kreb's-Ringer phosphate 20 ml.)

On the inspection of the free estrogen fraction, three fractions were found to decrease markedly, especially, the decrease rate of estriol fraction was com-

TABLE V. Estrogen in Fatty Tissue of Adult Female Rabbit. (μg per 100 g)

	Estrone	Estradiol	Estriol	Total estrogen
Free	6.20±2.42	5.50±2.95	4.06±1.65	15.78±5.72
Conjugated	3.26±0.23	2.26±0.17	1.41±0.08	6.94±0.35
Protein-bound	4.42±0.77	2.78±0.50	1.58±0.12	8.74±1.24

TABLE VI. Estrogen in Fatty Tissue of Oophorectomized Rabbit. (μg per 100 g)

	Estrone	Estradiol	Estriol	Total estrogen
Free	4.92±2.05	4.22±1.84	1.58±0.45	10.73±3.76
Conjugated	2.91±1.38	2.42±1.91	1.81±1.51	7.14±4.80
Protein-bound	3.92±1.64	2.14±0.39	1.65±0.32	7.46±1.94

paratively large.

b) Muscle : TABLE a VII and VIII showed the estrogen contained in muscles of 5 untreated and 4 oophorectomized rabbits. The average of free estrogen in the untreated muscle was 15.38±3.36 μg, conjugated 6.74±0.98 μg, and protein bound estrogen 8.48±0.69 μg. In the three estrogen types, free estrogens could be detected in the greatest amount in untreated muscles. Of the estrogen fraction, a considerably large amount of estrone was present, 58% in free estrogen, 47% in conjugated and 43% in protein bound estrogen. After oophorectomy, estrone and estradiol decreased apparently.

TABLE VII. Estrogen in Muscular Tissue of Adult Female Rabbit. (μg per 100 g)

	Estrone	Estradiol	Estriol	Total estrogen
Free	7.66±1.71	4.71±1.77	3.50±0.38	15.38±3.36
Conjugated	3.22±0.85	1.61±0.34	1.90±0.59	6.73±0.98
Protein-bound	3.98±0.19	2.22±0.26	2.92±0.48	8.48±0.69

TABLE VIII. Estrogen in Muscular Tissue of Oophorectomized Rabbit. (μg per 100 g)

	Estrone	Estradiol	Estriol	Total estrogen
Free	6.05±2.59	2.39±1.23	3.42±1.88	11.06±4.73
Conjugated	2.62±1.46	1.18±0.48	1.11±0.24	4.92±2.17
Protein-bound	2.87±1.59	2.12±1.76	1.45±0.92	6.44±4.25

c) Uterus : Of the estrogen contained in uterus of 5 untreated rabbits, free estrogen was found in the greatest amount with an average 19.52±3.32 μg and it occupied 51% of the total estrogen (TABLE IX). Of the estrogen fraction, the greatest amount of estrone was found, and it took 48% of free estrogen, 51% of conjugated estrogen and 43% of protein bound estrogen. After the operation free estrogen was extracted with a mean value of 15.32±3.85 μg. Thus, it appeared obvious that free estrogen considerably decreased after oophorectomy, but that the conjugated and the protein bound estrogen in uterus

showed negligible difference between the pre- and the post-operation. On the inspection of the free estrogen fraction, all of three fractions decreased a little, but in the other estrogen types, estrogen fractions were not reduced after the operation (TABLE X).

TABLE IX. Estrogen in Uterine Tissue of Adult Female Rabbit. (μg per 100 g)

	Estrone	Estradiol	Estriol	Total estrogen
Free	9.16±1.74	5.26±2.25	4.76±1.26	19.52±3.32
Conjugated	5.01±1.18	3.35±1.25	1.52±0.32	9.87±2.78
Protein-bound	3.77±0.94	2.49±0.19	2.57±0.40	8.83±0.73

TABLE X. Estrogen in Uterine Tissue of Oophorectomized Rabbit. (μg per 100 g)

	Estrone	Estradiol	Estriol	Total estrogen
Free	8.24±2.39	3.57±0.44	3.48±1.49	15.32±3.85
Conjugated	5.41±1.79	3.09±0.54	3.21±0.55	11.52±2.47
Protein-bound	5.00±2.75	3.35±1.31	2.11±0.54	10.47±4.32

d) Kidney: The greater part of estrogen contained in the kidney of untreated rabbits was free estrogen, it was extracted 18.63±3.29 μg on an average, but conjugated and protein bound estrogen were found in about a half amount of free estrogen. In regards to the estrogen fraction, estrone could be detected in the greatest deal in each estrogen type together, it took 43 % of free, 50 % of conjugated and 53 % of protein bound estrogen. In the second place of estrone, estriol fraction was proved in a large quantity in each estrogen type, and it took 30% of free, 27 % of protein bound estrogen (TABLE XI). After oophorectomy an average value of free estrogen was 15.61±3.54 μg, showed the decrease of 16 % as compared with the pre-operation level, but conjugated and protein bound estrogen did not show the decrease of a significant difference. Estrone and estriol fraction were reduced a little, but estradiol fraction did not show a decrease (TABLE XII).

TABLE XI. Estrogen in Renal Tissue of Adult Female Rabbit. (μg per 100 g)

	Estrone	Estradiol	Estriol	Total estrogen
Free	8.19±0.67	5.01±1.07	5.72±2.26	18.63±3.29
Conjugated	5.35±1.87	2.86±1.62	2.23±0.63	10.43±4.04
Protein-bound	5.46±0.78	2.03±0.22	2.83±0.50	10.35±0.93

Estrogen in fat, muscle, uterus and kidney decreased after oophorectomy; its majority was free estrogen, the decrease rate being 32% with fat, 28 % with muscle, 21.5 % with uterus and 16 % with kidney.

6) Investigation on the possibility of estrogen production in uterus and kidney.

After incubation with 20 ml of Krebs-Ringer phosphate solution and 2 g of

TABLE XII. Estrogen in Renal Tissue of Oophorectomized Rabbit. (μg per 100 g)

	Estrone	Estradiol	Estriol	Total estrogen
Free	6.44±2.02	5.68±1.37	3.53±0.47	15.61±3.54
Conjugated	5.51±2.81	2.53±0.96	1.45±0.39	9.41±2.61
Protein-bound	4.46±0.97	2.10±0.52	1.57±0.50	8.13±1.66

sliced tissue, estrogen was extracted with the mean value of 1.35 μg from uterus and 1.23 μg from kidney. These are the control for the following experiment. As the conditions of experiment, 1) group of addition of PMS 250 I.U, 2) group of addition of testosterone 1000 μg., 3) group of addition of testosterone 1000 μg and PMS. 250I.U., were undertaken.

a) Uterus: Fig. 7 is a diagram to illustrate the results of this study. On the first group, estrone of 0.64 μg was extracted, estradiol of 0.43 μg, estriol of 0.34 μg on an average, but showed no significant difference in comparison with the control. On the second group in which testosterone 1000 μg was added, estrone, estradiol and estriol could be detected in 1.21 μg, 0.55 μg and 0.88 μg respectively. Total estrogen increased in an amount of two times the control, so the fact mentioned above suggests that the conversion to estrogen from testosterone may occur in the uterus tissue.

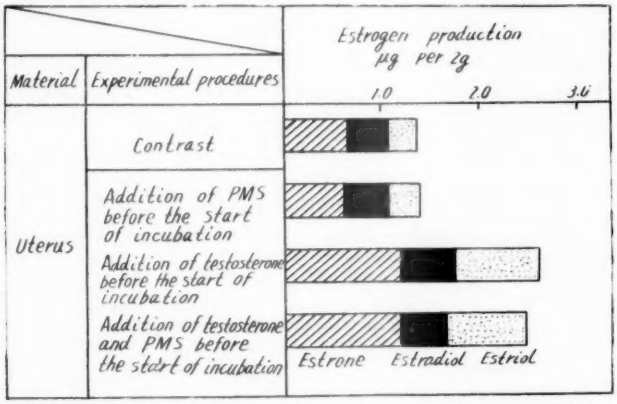

Fig. 7. Investigation on the possibility of estrogen production in the uterus of rabbit.

b) Kidney: The results are shown in Fig. 8. The total estrogen extracted in the first group was in the mean value 1.64 μg. Of the estrogen fraction, estrone of 0.90 μg, was extracted, estradiol of 0.39 μg, estriol of 0.35 μg, and estrone increased a little in comparison with the control. On the second group, estrone, estradiol and estriol were extracted with the mean values of 1.64 μg, 1.03 μg and 0.60 μg respectively. It was proved that the estrogen increased drastically more

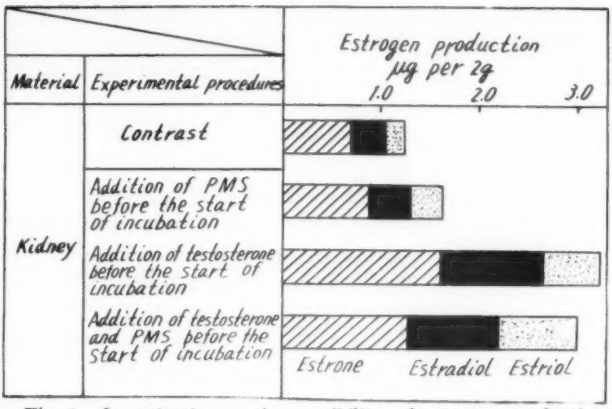

Fig. 8. Investigation on the possibility of estrogen production in the kidney of rabbit.

Figs. 7, 8. hatched bar : estrone ; shaded bar : estradiol; stippled bar : estriol.

than the control. On the third group, estrone, estradiol and estriol could be detected with the mean values of 1.32 μg, 0.88 μg and 0.81 μg respectively, but no significant difference was found in comparison with the second group.

DISCUSSION

It is known already that urinary estrogen excretion was uniformly low in the oophorectomized and climacteric woman, but quite a high proportion of the women continued to excrete estrogen after oophorectomy and climacterium.[12][13] Dao & Smith[14] and Emerson[15], using a biological assay, have detected estrogen in the urine of oophorectomized women. My study demonstrated that the average value of 39.22 $\pm$20.17 μg per 24 hours was detected in the urine of climacteric women, and in comparison with the normal estrogen excretion it was a larger quantity and there were some individual differences. Of the estrogen fraction, estradiol fraction was found in a large quantity in the urine of women with uterus cancer, it was due to the metabolic abnormality of estrogen and this was in accord with the finding of Ayre,[16] Nakayama[17] and Koga.[18] Estrogen excretion decreased shortly after oophorectomy. However, in 6 of 10 patients, estrogen increased at once after that, especially, estrone could be detected obviously. It is possible that the estrogen excretion increased in patients shortly after oophorectomy was due to the stress of operation ; consequently it seemed to increase the estrogen secretion from the adrenal cortex.

The extra-ovarian source of estrogen is thought to be the adrenal gland. Estrogen excretion shows a transient rise after the injection of corticotrophin in oophorectomized women[19]. After removal of both ovaries estrogen excretion

had decreased from day to day, but in some case a comparatively large quantity of estrogen could be detected in the urine. Though there were several methods of estimating the pure estrogens, they are not directly applicable to accurate estimation of estrogen in rabbit urine or tissue because of the presence of impurities and microgram quantities of the natural estrogens. Matsumoto and Seki[20] have shown the estimation in urinary estrogen of pregnant rabbits, showed that the estrogen excretion occurred in a small amount and that estradiol fraction took a greater part of estrogen. In this test urinary estrogen excretion of rabbit decreased markedly after oophorectomy. Of the estrogen fraction, the decrease rate of estrone was lower than that of the other fraction level. I have extracted the estrogen of 3.49 ± 0.62 μg per 24 hours with an average value from untreated rabbit urine, after oophorectomy in animals estrogen excretion indicated the same tendency as that of the oophorectomized women.

In further adrenalectomized case, estrogen was reduced from the mean value of 1.21 ± 0.82 μg to 0.43 μg in three specimens, and a small amount of estrogen could be detected in the urine. However, it is not concluded yet that the estrogen excreted after oophorectomy-adrenalectomy was either due to the conversion of DOCA administered or to the presence of accessory adrenal tissue or to the discharge of estrogen retained in organ tissues. Dao, Huggins, and West and co-workers[19], in studies of women with breast cancer, detected estrogens in the urine after bilateral oophorectomy, but they could not find estrogens after combined oophorectomy-adrenalectomy. However, Bulbrook, Greenwood and Williams[21] detected estrogens in the urine in five of seven oophoremy-adrenalectomy women. Shibusawa[22] indicated that in one of ten cases estrogen excretion fell to zero after oophorectomy-adrenalectomy, in the other cases could be detected the mean value of 10.5 μg per 24 hours and in some cases estrogen excretion was found to increase 30 days after the post-operation.

After adrenalectomy accessory adrenal tissue becomes hypertrophic; it seems to be sources of the estrogen secreted in some women after adrenalectomy. I only measured urinary estrogen till 12 days after the oophorectomy-adrenalectomy, so could not conclude that the estrogen excreted in the urine after the operation was secreted from accessory adrenal tissue. The estrogen contained in the organ tissue seems to affect the estrogen excretion after the removal of ovary. The isolation and estimation of estrogens in organ tissues have been made with placenta, ovary and adrenal cortex. Albert[23] reported that fatty tissue is important one which retains shortly hormones and that the hormones could be extracted from mammary gland, skin and muscle. It is reported that a great quantity of hormone is retained in liver, kidney, fat and muscle. Akiyama[24] has proved that estrogen was retained in fat, kidney, muscle and lung. I extracted a comparatively larger deal of estrogen from fat, muscle, uterus and kidney ; the contained estrogen was found to decrease after oophorectomy, especi-

ally, the free estrogen decreased markedly in each organ. The decrease rate of free estrogen in fat tissues was 32%. It seems that estrogen is fat-soluble, so the estrogen retained in organ tissue, especially, fatty tissue is discharged in compensation for lack of estrogen after oophorectomy-adrenalectomy, it keepts the action of estrogen in blood and makes estrogen work continuously. Although many theories of the estrogen production have been propounded, they have not yet been established. At present the conversion of testosterone to estrogen is appreciated experimentally and clinically. Yamada[25] found the conversion of testosterone to estradiol after incubation with rat ovary slices together with gonadotrophin. There exist a considerable literature on the estrogen production, little is known of the experiments showing possibility of estrogen production in uterus and kidney.

In this test I found that in the group added PMS only, estrogen did not increase as comparison with the control in uterus and kidney, but in the group with testosterone 1000 μg added estrogen increased whether PMS was added or not, particularly in uterus estrone and estriol fraction increased markedly, and in kidney three fractions indicated a highly significant increase. However, there was no significant difference between the group with PMS in addition to testosterone and that without it. On the swine ovary, estradiol production by the conversion of testosterone has been proved to increase after addition of PMS,[25] so it could be concluded that the estrogen production in uterus and kidney did not occur. It appeared obvious that the estrogen retained in uterus and kidney was discharged after removal of the endocrine source to compensate for the lack of the hormone and that at this time one part of testosterone retained in organs was converted to estrogen.

SUMMARY

1) Urinary estrogen excretion of climacteric women with uterus cancer was found in a comparatively large quantity and there were individual differences.

2) Urinary estrogen excretion of climacteric women after post-oophorectomy decreased temporarily, increased afterwards, and 12 days leter the post-operation estrogen was found to decrease again. Of the estrogen fraction, the decrease of estradiol was the clearest.

3) The greater part of estrogen extracted from the rabbit urines was about estradiol fraction. The mean value of estrogen excretion 28 days after oophorectomy was about a half of the pre-operation level. After adrenalectomy besides, estrogen decreased apparently, but a small amount of estrogen could be detected now in the urine.

4) Blood estrogen of adult female rabbits indicated the mean value of 2.35 ± 0.24 μg per serum 10 ml, after oophorectomy decreased to 1.56 μg and there were significant differences. However, the decrease rate of free and protein bound

estrogen was about the same.

5) In this test it was affirmed that there were comparatively large amounts of estrogen in rabbit brain, lung, liver, kidney, bowel, uterus, muscle and fat.

6) The estrogen contained in rabbit fat, muscle, kidney and uterus decreased markedly after oophorectomy, especially, the decrease of estrogen contained in fat was most prominent.

7) It was concluded that no estrogen production occurs in rabbit uterus and kidney, but that the conversion of testosterone to estrogen occurs.

Grateful acknowledgment is made to Prof. K. Kushima and Assist. Prof. M. Suzuki for their continued interest and guidance in this investigation.

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Experiments on the Chemotherapeutic Treatment of Carcinoma with Citral and Citronellal and those Combined with PCMB

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At the last annual meeting[1] (December 18, 1960) of the Japan Cancer Society, we reported on changes of fine structure of tumor cells suffered by the application of several cancer chemotherapeutic remedies. We demonstrated many electron micrographs concerning animal experiments on this problem. There is a remarkable contrast seen between Citral and PCMB (p-chloro-mercuri-benzoic acid.) The former causes changes of nuclear elements (nuclear membrane, chromatin granules and nucleolus) as well as cytoplasm (ergastoplasmic reticulum and ground substance), while the mitochondria are relatively well reserved, although not free from change. On the contrary PCMB causes a degeneration of mitochondria in a high degree, but not much of the other elements. Accordingly we suggested combined use of Citral and Citronellal with PCMB in cancer chemotherapy.

In this investigation we used C3H/dd. F_1 mice. We have experienced these two years that C3H MH134 (adeno-carcinoma) is quite sensitive to aldehyde. We are indebted to Dr. H. Sato, formerly professor of Pathology of Fukushima Medical College and now professor of the Research Institute for Tuberculosis and Leprosy of Tohoku University of Sendai, for his kind advice and providing of the strain of mice.

EXPERIMENT I

On December 11, 1959 a lot of mice C3H-F_1 of about 20 g were inoculated into the tail subcutaneously with 0.05 cc. of diluted tumor ascites of MH134 (cells were not counted). The animals were divided in three groups.

Group I 9 mice were given 1/52 cc, 10% d-Citronellal in olive oil per os every day.

Group II. 9 mice were given dl-Citronellal in the same way as Group I.

Group III. 10 mice served as control.

大里俊吾，森　一，森田倫雄

The administration of the remedies was begun on the same day as inoculation. On Dec. 23, 1959, the 11th day of inoculation, the tails of animals were cut off at the root. The animals continued to live, many of them getting metastatic tumor in the retroperitoneal lymph nodes.

Fig. 1 shows survival duration of these animals. Among 9 animals of Group I, 2 died within 50 days, 3 died after some 60 days. One died about 175 days after inoculation. And 2 survived 190 days, when they were killed.

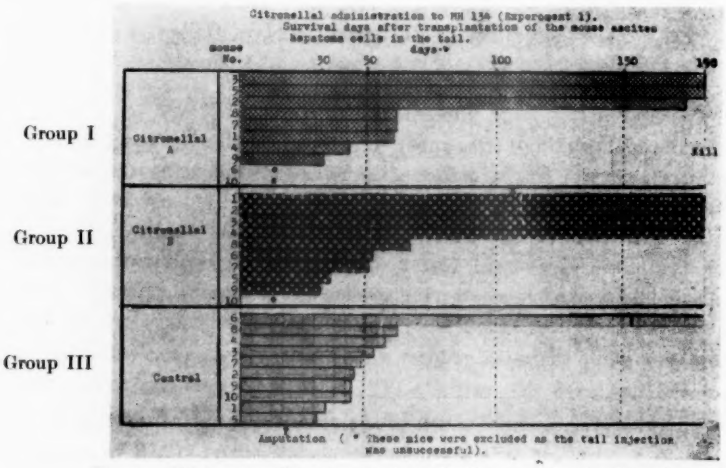


Fig. 1. Life's span of each animal of the 3 groups in the Experiment I.

Of the Group II, 2 died some 30 days after inoculation. 3 died between 50 and 70 days. And 4 survived 190 days after inoculation.

Among 10 control animals (Group III) 9 died within 60 days after inoculation and one survived 190 days and then killed.

EXPERIMENT II

A lot of mice were inoculated with diluted ascites of MH134, cells were not counted, on March 5, 1960. Anticancer remedies were administered on and after the same day. They were divided in 5 groups.

Group I. 8 animals received 1/52 cc. of 10% d-Citronellal in olive oil per os every day.

Group II. 8 animals received the same dose of dl-Citronellal per os every day.

Group III. 8 animals received Chromomycin 0.2γ intraperitoneally every other day.

Group IV. 8 animals received dl-Citronellal and Chromomycin as above.

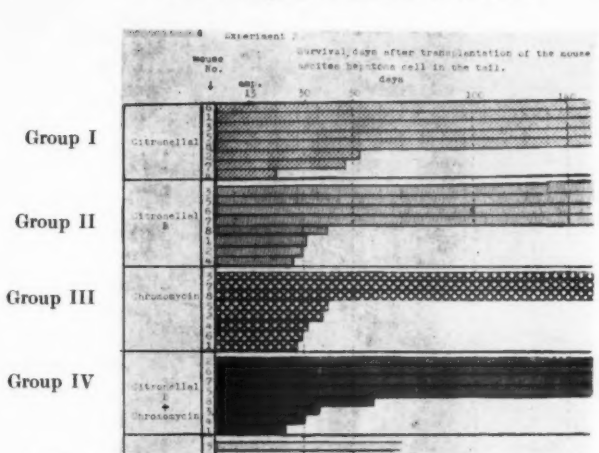


Fig. 2. Life's span of each animal of the 5 groups in the Experiment II.

Table I. Macroscopic Aspects (Photos) of the Tail of Each Animal of the 4 Groups Cut Off on the 19th Day after Inoculation of Tumor in the Experiment II.

Citronellal administration to MH 134. (Experiment II)

Comparative figures of the tumor growth in the tails which were cut in 18 days after inoculation of tumor cells.

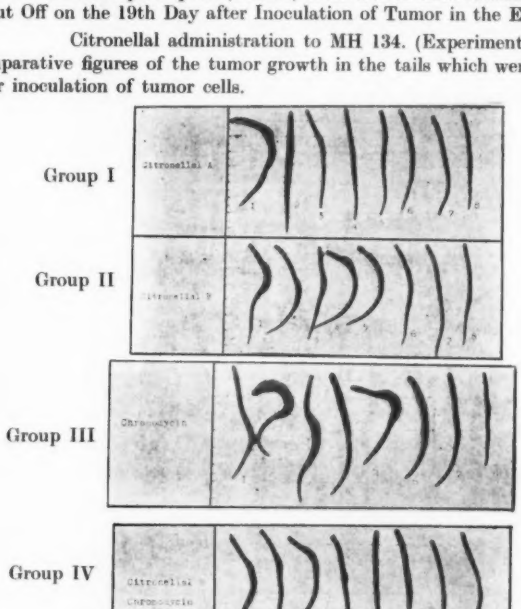
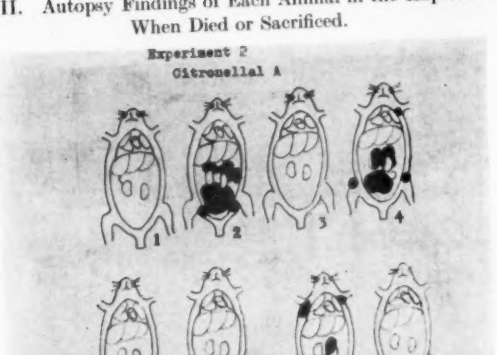


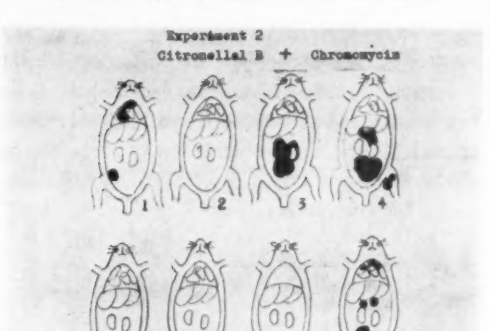
Table II. Autopsy Findings of Each Animal in the Experiment II, When Died or Sacrificed.

Group I

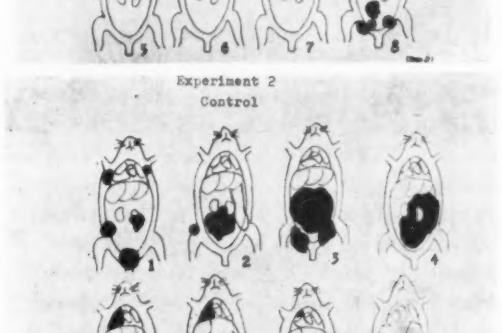


Group II

Group III



Group IV



Group V

Group V. 8 animals. Control.

On the 18th day of inoculation the tails were cut off at the root.

Fig. 2 shows durations of life in all of the animals. In Table I, macroscopic aspects of the tails are given. As we see in it, animals of treated groups had in general less tumefaction of the tails.

There were some surviving animals among each treated groups, when they were sacrificed in Sept. 11, 1960, 190 days after inoculation. Group I (d-Citronellal) 5 ; Group II (dl-Citronellal) 4 ; Group III (Chromomycin) 3 ; Group IV (Chromomycin + dl-Citronellal) 4 ; Group V (Control) O.

Table II shows autopsy findings of all groups. Animals free from tumor ; Group I (d-Citronellal) 5 ; Group II (dl-Citronellal) 4 ; Group III (Chromomycin) 3 ; Group IV (dl-Citronellal) + Chromomycin) 4 ; Group V(Control) O.

From the Experiment II, consulting the Experiment I, we would be able to conclude a remarkable sensitivity of MH134 to Citronellal. It seems that there is little difference between d-Citronellal and dl-Citronellal in their efficacy on MH134

tumor. As we described in the previous report, Chromomycin causes somewhat more obvious changes in mitochondria than Citronellal, while the latter is effective on other elements (nuclear membrane, nucleolus, chromatin substance; ergastoplasmic reticulum ground substance of cytoplasm). So we made combined use of Citronellal and Chromomycin with great expectation. Chromomycin itself was really effective but less than Citronellal. Dl-Citronellal+Chromomycin seems to be not more strongly active than dl-Citronellal alone.

EXPERIMENT III

On Dec. 7, 1960 a lot of mice of C3H/dd F1 of about 23 g were inoculated and divided into 6 groups.

Group I, 11 mice, d-Citronellal; Group II, 11 mice, dl-Citronellal; Group III, 10 mice, dl-Citronellal+PCMB; Group IV, 10 mice, Citral+PCMB; Group V, 10 mice PCMB;[3] Group IV, 10 mice, Control. Administration of remedies begun on the 3rd day of inoculation. Citronellal 1/52 cc. of 10% dilution in olive oil. 0.05 cc of 5% emulsion of Citral was injected into the muscle. Citronellal and Citral were given every day. PCMB was injected into the muscle on the 5th day and 7th day. It was used as 10^{-3} mol. in n/10 NaOH made pH7.3 by n-HCl. The dose was 0.2 ml at one time.

For the inoculation of tumor MH134, ascites fluid of the 5th day of the inoculation of tumor into peritoneal cavity, diluted 1 : 4 with sodium citricum, was used, and injected in the tail at 1/3 site from the root. On the 8th day the

Fig. 3. Life's span of each animal of the 6 groups in the Experiment III.

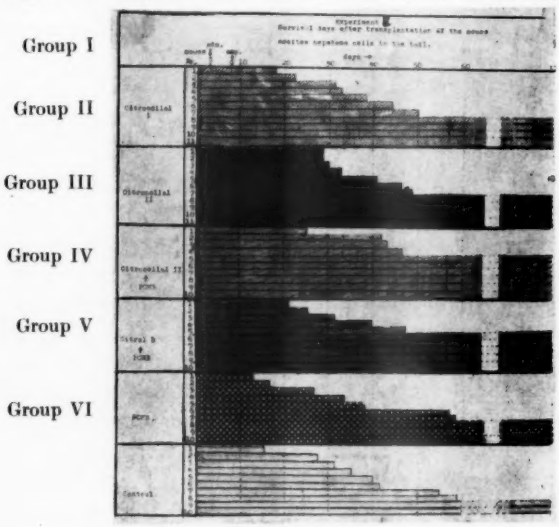
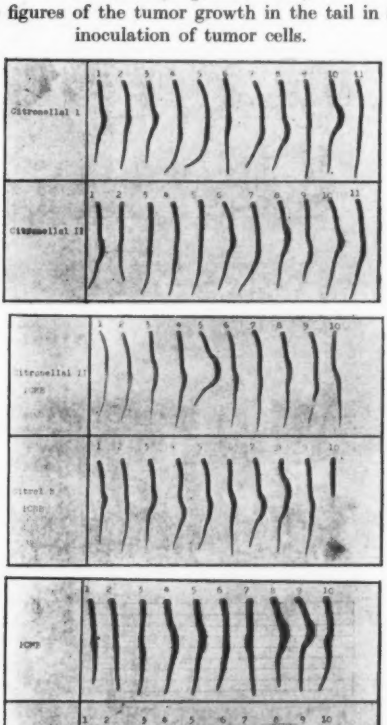


Table III. Macroscopic Aspects (Photos) of the Tail of Each Animal Cut Off on the 8th Day after Inoculation of Tumor in the Experiment III.

MH 134. (Experiment III)
Comparative figures of the tumor growth in the tail in 8 days after inoculation of tumor cells.



tails were simultaneously cut off at the root.

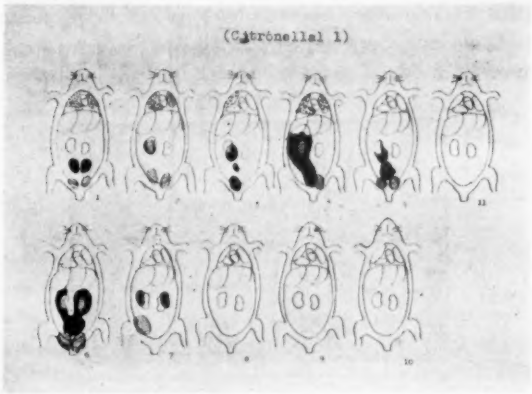
Fig. 3 shows the life's span allotted for the animals of each group. Table III shows the macroscopic photos of the tails. All the animals had more or less tumefaction of the tails at the iniection site. Some difference between the groups could be detected if carefully examined, but we did not go further in it.

At the end of March 1961, 114 days after inoculation, the surviving animals were sacrificed.

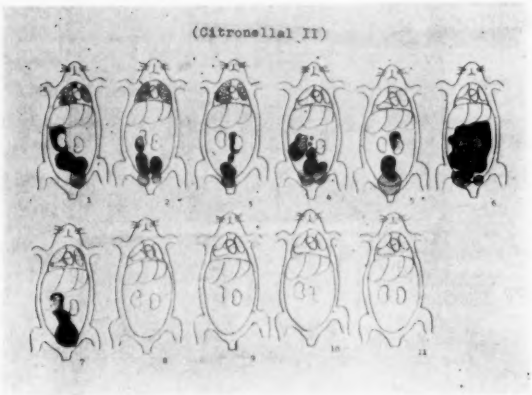
Group I (d-Citronellal) 4; Group II (dl-Citronellal) 4; Group III (dl-Citronellal +PCMB) 6; Group IV (Citral + PCMB) 5; Group V (PCMB) 3; Group VI

Table IV. Autopsy Findings of the Animals at Their Death or When Sacrificed.

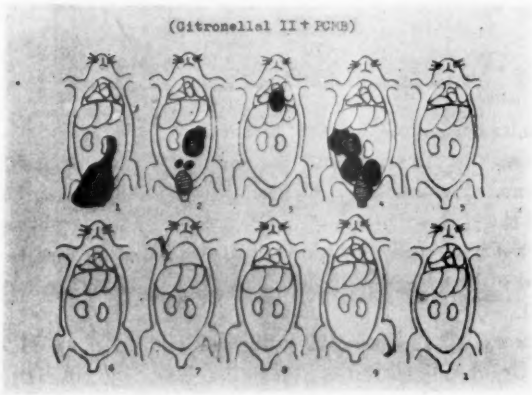
Group I



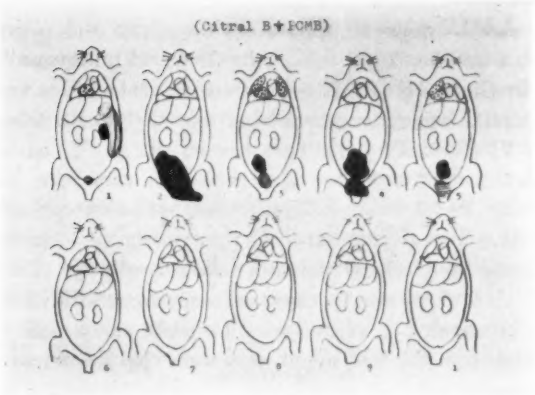
Group II



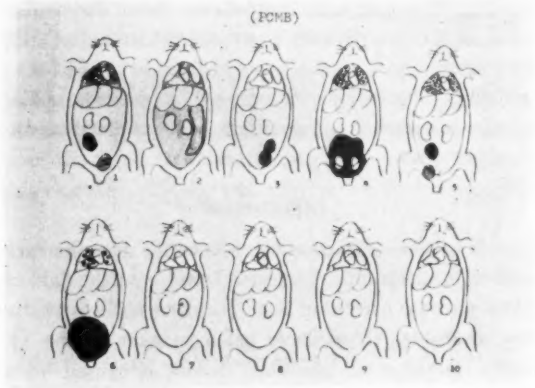
Group III



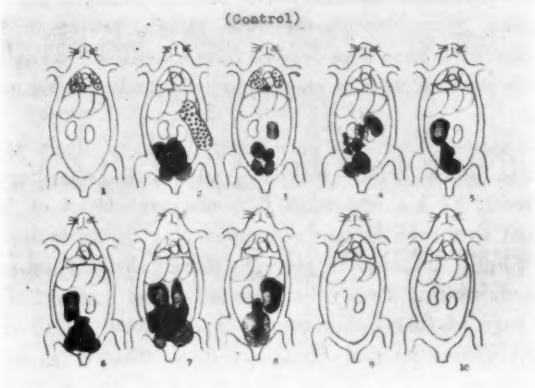
Group IV



Group V



Group VI



(Control) 2.

Table IV shows autopsy findings of all animals of each group: In Group I (d-Citronellal), 4 are free from tumor; in Group II (dl-Citronellal), 4 are free from tumor; in Group III (PCMB+dl-Citronellal), 6 free from tumor; in Group IV (PCMB+Citral): 5 free from tumor; in Group V (PCMB), 4 free from tumor; and in Group VI (Control), 2 free from tumor.

It is to be noticed in this place, that there were some mice, which had white blotch on the tail. In the Group VI (control group) there were 3 with white blotch or spot among 11. Two of them survived the experiment. Group II: 2, Group III: 1, and Group IV: 1 ,these had such tails respectively. They survived for the most part. It is very probable that such a mouse as with white blotch on the tail may have inherited some character of the white mouse, and that those mice with white blotches on the tails might have somewhat greater resistance against MH134 tumor.

Notwithstanding the circumstance, judging from above data it would be quite evident that PCMB is a good co-operator of Citronellal and may be also of Citral. For the time being we do not know the mechanism of the co-operation of PCMB to Citronellal. The results of our electron-micrographic investigation[1] of tumor cells under anticancer agents might be considered as an explanation of the mechanism.

DISCUSSION

It is tendency in chemotherapeutic researches into bacterial infection to select remedies of wide spectrum. The same trend is in the field of cancer chemotherapy. For this purpose screening tests are done with earnestness. Of course it would be very desirable, if we could get a panacea in the chemotherapy of tumors. Generally speaking an infectious disease is caused by a foreign body, (bacteria, protozoa, or virus) invading from outside. Cancer is quite different from infectious diseases. Some tumors are, it is known, proved to be viral. But carcinoma is believed to have been derived from a soma cell: so called soma mutation. From this point of view, a carcinoma cell stands in very near relation to the soma cell.

A remedy against carcinoma cells might be very likely to be more or less inimical to soma cells. Makino[4] advocated astem-line-cell theory of cancer as a result of his researches into the karyotypes of the tumor cell. He is of opinion that each human carcinoma may be different from others in origin. Here is much difficulty in getting a cancer chemotherapeuticum of wide spectrum without injuring soma cells, especially bone marrow. Osato[4], one of us, concluded from his chemotherapeutic experiences[5][6] of 15 years in human carcinoma, that Citronellal and Citral are most effective in adenocarcinoma. His clinical experience taught us that Citronellal and Citral were not good for the

treatment of sarcoma, leukemia or Hodgkin's disease. Citronellal and Citral are quite efficacious against types of carcinoma other than adenocarcinoma. But Osato has never seen consecutive healing in skirrhus and mucinous carcinoma owing to Citronellal and Citral. Fortunately enough a patient stands long administration of these remedies and does not show leukopenia. as seen by other anticancer agents. From all these, it would be quite natural, if we call Citral and Citronellal anticancer remedies of narrow spectrum. Combined use of Citronellal and Citral with other anticancer agents would be preferable, if amore conspicuous effect is desired. Our previous paper as well as the present have been started from this stand point. Animal experiments with combined use of Aldehydes and PCMB seem to be quite promising. As a next step, it must be verified in the clinical use, the result shall follow the present paper.

SUMMRAY

1. In the present paper, animal experiments of the chemotherapy with Citronellal in MH134 (adenocarcinoma) of C3H F1 strain are reported.

2. About 30 to 50% of tumor animals were cured when the chemotherapy with Citronellal was started on the same day as inoculation of tumor.

3. When the administration of Citronellal was begun on the third day of tumor inoculation, the result of chemotherapy was lower, yet the effect was quite obvious. In this case the efficacy was very much promoted with the combined use of PCMB.

4. Our expectation in the co-operation of PCMB with Citronellal and Citral, based upon the electron microscopic study of tumor cells mentioned in the previous paper, is verified in this report.

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Salivary Lysozyme and ts Relation to Angular Stomatitis in Ariboflavinosis

Part III. Morphological Changes of Epithelial Cells of Lips after Incubation with Lysozyme

By

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Though angular stomatitis has been accepted as one of clinical manifestations of ariboflavinosis, still remains obscure the mechanism by which angular stomatitis is brought about.

Preceding papers of mine[1][2] revealed the following results; lysozyme activity showed no significant difference in saliva from subjects with or without ariboflavinosis, but micrococcus lysodeikticus became easily susceptible to lytic action of lysozyme when the microorganism had been cultured on riboflavin-free media.

In the present paper I will show that epithelial cells obtained from the lip are more easily susceptible to lytic action of lysozyme in the cases with ariboflavinosis than in those without ariboflavinosis.

MATERIALS AND METHODS

A small collection of epithelial cells was obtained from lips of children, by scratching several times the central part of the lower lip with a surgical spoon, and suspended in a few cc (— of course, the amount being equal in the test and control cases —) of physiologic saline. To 1.0 cc of this suspension was added 0.1 cc of 5 mg% solution of lysozyme, and the content was mixed well and kept at 37°C for 5 days. Before and after the incubation, morphological changes of the epithelial cells were examined by a phase contrast microscope. According to the morphological findings each of the epithelial cells was classified into 3 types (Types I, II and III).

Type I: Normal.

Type II: Both nucleus and cell became irregular or atrophic in contour, and mitochondria became larger in size and decreased in number.

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Type III: Pycnotic nucleus was surrounded by mitochondrias of larger size, and many vacuoles were seen in cytoplasm. Nuclei were in some cases almost completely destroyed, their remains being recognized only as mere specks.

The children subjected to this investigation consisted of 2 groups: Group I were 15 children free from ariboflavinotic signs, and Group II were 16 children with ariboflavinosis.

RESULTS AND DISCUSSION

The results were shown in Tables I and II (cf. Tables I & II). Before the incubation of the epithelial cells with lysozyme, the distribution of Types I, II and III of these epithelial cells was 70.7%, 14.6%, and 14.7% in Group I respectively, while it was 74.8%, 17.1% and 8.1% in Group II respectively. On the 5th day of the incubation, the distribution of the Types showed a remarkable difference between Groups I and II, that is to say, Types I, II, and III were of 58.2%, 21.5%, and 20.0% in Group I respectively, while they were of 15.1%, 16.1%, and 58.3% in Group II respectively.

These results revealed that lysozyme exerted its lytic action more conspicuously upon labial epithelial cells from ariboflavinotic children than upon

Table I. Morphological Changes of Epithelial Cells of Lips after Incubation with Lysozyme, Group I.

No. of case	Marks for B_2-deficiency[2]	Distribution of types of epithelial cells (in number)					
		Before incubation with lysozyme			5 days after incubation with lysozyme		
		Type I	Type II	Type III	Type I	Type II	Type III
1	0	55	15	18	28	23	16
2	0	25	4	2	29	8	13
3	0	24	5	2	14	8	4
4	0	33	3	13	33	4	16
5	0	20	6	6	18	8	12
6	0	23	3	3	24	14	12
7	0	12	2	5	34	8	6
8	0	11	3	2	12	4	6
9	0	9	2	3	14	7	11
10	0	18	5	2	18	5	6
11	0	23	4	5	15	4	2
12	0	53	12	8	43	13	6
13	0	18	7	2	18	6	2
14	0	27	6	4	26	7	3
15	0	32	2	5	83	16	8
Total in number		385	79	80	364	135	123
%		70.7	14.6	14.7	58.5	21.5	20.0

TABLE II. Morphological Changes of Epithelial Cells of Lips after Incubation with Lysozyme, Group II.

No. of case	Marks for B_2-deficiency[2]	Distribution of types of epithelial cells (in number)					
		Before incubation with lysozyme			5 days after incubation with lysozyme		
		Type I	Type II	Type III	Type I	Type II	Type III
1	4	38	6	2	8	6	33
2	1	32	7	0	4	2	17
3	4	56	11	3	0	0	17
4	4	25	5	6	8	13	52
5	4	12	6	10	3	1	10
6	2	38	2	0	3	4	13
7	4	23	5	2	7	7	16
8	3	21	4	1	12	6	39
9	1	36	12	5	2	7	30
10	3	7	5	4	3	7	20
11	4	65	17	8	5	10	23
12	5	18	6	2	7	5	16
13	4	54	12	4	10	8	25
14	5	5	4	2	0	1	2
15	6	20	3	1	0	1	11
16	5	14	1	0	2	8	10
Total in number		464	106	50	74	81	334
%		74.8	17.1	8.1	15.1	16.1	58.3

those from healthy children. This finding seemed to be in good accord with my previous finding[2] that micrococcus lysodeikticus grown on riboflavin-free media succumbed more easily to lytic action of lysozyme.

Basing upon the results above mentioned, I believe we can explain the pathogenesis of angular stomatitis in ariboflavinosis as follows:- Riboflavin deficiency thus causes the epithelial cells of lips to easily succumb to lytic action of salivary lysozyme, leading to a maceration of lips, which will play an important role for development of vertical fissures at the central part of lips and of transverse fissures at oral corners (angular stomatitis).

CONCLUSIONS

Epithelial cells of the lips obtained from ariboflavinotic children succumbed more easily to lytic action of lysozyme than those from healthy children. It was suggested that this might be one of contributing factors for development of a maceration of labial epithelial cells at oral corners—angular stomatitis—in ariboflavinosis.

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A Method for Colorimetric Estimation of Peroxidase in Serum or Plasma

By

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About 30 years ago the late Dr. T. Arakawa[1] from Our Laboratory reported an exceedingly sensitive peroxidase reaction for human milk. As was shown in his report, the Arakawa reagent is innert to normal *human serum.*

With a slight modification of the Arakawa reagent, we devised a method for colorimetric estimation of peroxidase in serum or plasma (*α*-Napthol-Benzidine method of Our Own).

Reagents required

R. 1. M/15 phosphate buffer of pH 5.29

R. 2. Diluted Hayem's solution: Original Hayem's solution (1.0 g of NaCl, 5.0 g of Na_2SO_4 and 0.5 g of $HgCl_2$ are dissolved in 200 cc of distilled water) is added with distilled water in the ratio of 2 : 1.

R. 3. *α*-Naphthol-benzidine solution: 0.2g of *α*-naphthol and 0.2 g of benzidine are dissolved in 100 cc of acetone. This is prepared freshly before use.

R. 4. Hydrogen peroxide solution: 2.0 cc of 0.1% solution of hydrogen peroxide are added with acetone up to 100 cc. This is freshly prepared before use.

R. 5. Ethyl acetate

Procedure

0.4 cc of serum or heparinized plasma is put into a test tube containing 2.0 cc of the diluted Hayem solution (R. 2), and 6.0 cc of the phosphate buffer (R. 1) are added. 1.0 cc of the *α*-naphthol-benzidine solution (R. 3), and then 2.0 cc of the hydrogen peroxide solution (R. 4) are added, and the content is well mixed and kept in a water bath of 23°C for 30 minutes. After the incubation 5.0 cc of ethyl acetate (R. 5) are added and the content is mixed throughly, the ethyl acetate layer is separated from the aqueous layer. Any turbidity of the ethyl acetate layer should be avoided. A clear filtrate will be easily obtained if filtered through a dry filter paper. The clear pink color of the

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ethyl acetate layer is measured electrophotometrically at 500 mμ.

Preparation of standard curve: Pure preparation of horseradish peroxidase (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.) is used for preparation of standard curve (cf. Table I & Fig. 1).

TABLE I. Relation Between Amount of Horseradish Peroxidase and Extinction in the α-Naphthol-Benzidine Method (Our Own Method).

Amount of horseradish peroxidase (γ)	Extinction × 10^3 at 500 mμ
0.005	11
0.01	24
0.015	39
0.02	54
0.03	75
0.04	95
0.05	120
0.06	150

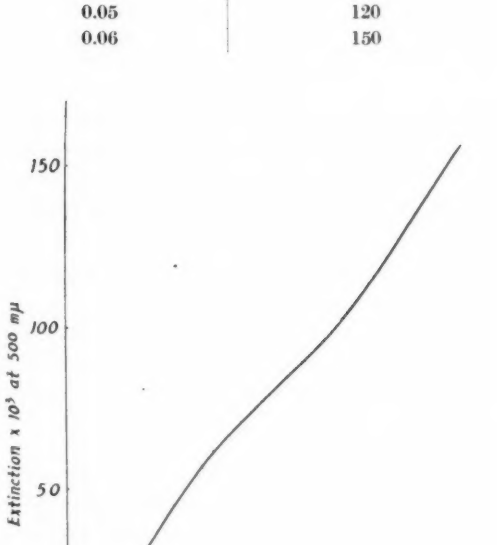

Fig. 1. Relation between amount of horseradish peroxidase and extinction in the α-naphthol-benzidine method (Our Own Method)

RESULTS

Optimal pH :– The maximal intensity of the color developed at pH 5.29 (cf. Table II).

TABLE II. Influence of pH upon the α-Naphthol-Benzidine Reaction (Our Own Method).

pH of M/15 phosphate buffer	Extinction $\times 10^3$ at 500 mμ
4.53	289
5.29	335
5.59	275
5.91	160
6.24	92
6.47	86
6.64	42
6.81	37
6.98	21
7.17	16
7.73	0

Influence of contamination with hemoglobin: Hemoglobin solution did not interfere with our own method below the concentration of 6 mg%. When a specimen of serum happened to be macroscopically as being hemolyzed, the concentration of hemoglobin in the serum amounted to 10 mg% at least. 0.4 cc of 10 mg% solution of hemoglobin gave an extinction of 7 in this reaction, so the specimens with macroscopic hemolysis should not be subjected to the present estimation.

Peroxidase content in plasma from normal children :– Plasma peroxidase was found $59.3 \times 10^{-3}\gamma$/cc in an average of 12 girls at the age of from 7 to 11 years and $58.7 \times 10^{-3}\gamma$/cc in an average of 30 boys at the same range of age (cf. Table III).

DISCUSSION

In 1926 Kwasniewski and Henning[2] reported that a positive peroxidase reaction in serum was found only in some of the cases with myeloid, lymphoid leukemia or leukocytosis. For the detection of the peroxidase in serum, these authors adopted such a method that, to 0.5 cc of serum, 0.5 cc of the peroxidase reagent of Schmorl[3] (H_2O_2 + Benzidine in aqueous solution) was added, and a development of brown color after incubation at 37°C for 24 hours was designed as an evidence of the positive reaction for peroxidase in serum.

In 1951 Koch and Mechow[4] investigated an occurrence of the remains of neutrophils and its relation to peroxidase reaction in serum, especially in patients

TABLE III. Peroxidase Content in Plasma from Healthy Children (Our Own Method).

No. of case	Age (years)	Sex	Peroxidase content in plasma $\gamma \times 10^{-3}$/cc	No. of case	Age (years)	Sex	Peroxidase content in plasma $\gamma \times 10^{-3}$/cc
1	7	Girls	55.8	13	7	Boys	53.2
2	7		66.2	14	7		70.0
3	7		42.0	15	7		50.0
4	8		87.5	16	7		70.0
5	9		51.8	17	7		50.8
6	9		62.0	18	8		40.8
7	9		65.0	19	8		47.5
8	10		45.8	20	8		40.8
9	11		55.8	21	8		65.0
10	11		62.0	22	8		60.8
11	11		53.3	23	8		65.0
12	11		65.0	24	8		50.8
Average			59.3	25	9		62.0
				26	9		77.5
				27	9		62.0
				28	9		50.8
				29	9		55.8
				30	9		73.6
				31	10		50.8
				32	10		50.8
				33	10		73.7
				34	10		83.2
				35	10		59.2
				36	10		53.3
				37	11		58.8
				38	11		47.5
				39	11		57.5
				40	11		60.8
				41	11		55.8
				42	11		66.3
				Average			58.7

receiving hormone therapy of various kinds. The method adopted by these authors was the benzidine-H_2O_2 method which was almost the same as used by Kwasniewski and Henning in 1926.

In our own method reported in this paper, the resultant color for colorimetry was very stable and of the maximal absorption at 510 mμ. The stability of the color was due to combined use of α-naphthol with benzidine and the extraction of the color into ethyl acetate.

The standard curve in our own method was prepared from a pure preparation of horseradish peroxidase, so the peroxidase content in plasma or serum was provisionally expressed in an absolute amount of the horseradish peroxidase. According to Agner[5] the horseradish peroxidase was 10~20 times as potent as

verdoperoxidase. When a pure preparation of verdoperoxidase will come to be available for us in future, peroxidase in serum or plasma will be naturally expressed in an absolute amount of verdoperoxidase.

In 1958 Bünte and Demling[6] reported a colorimetric method, using pyrogallol reaction, for quantitative estimation of peroxidase in serum, and they were able to establish the existence of peroxidase in normal serum and an increase of serum peroxidase in myeloid leukemia.

We used Bünte's method and our own for peroxidase estimation of the same specimens of serum and cow's milk. The standard curve for each method was prepared by using a standard horseradish peroxidase specimen. As will be seen from Table IV, peroxidase content in serum was found to be much higher by

Table IV. Comparison of Peroxidase Content in Sera or Cow's Milk by Bünte and Demling's Method with That by Our Own Method.

Specimens	Peroxidase content (γ/cc)	
	By Bünte & Demling's Method	By Our Own Method
Serum A	1.8	0.48
Serum B	1.6	0.28
Serum C	3.2	0.37
Cow's Milk	3.5	16.5

Bünte-Demling's method than by our own method, while in case of cow's milk peroxidase content was found to be lower by Bünte-Demling's method than by our own method. At the present time this discrepancy between the results by Bünte-Demling's method and those by our own method remains unsolved.

CONCLUSIONS

A simple colorimetric method for quantitative estimation of peroxidase in serum or plasma was described.

The averages for the plasma peroxidase, expressed as horseradish peroxidase, were 59.3×10^{-3} γ/cc for healthy girls and $58.7\times10^{-3}\gamma$/cc for healthy boys of the age of from 7 to 11 years.

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Visual Learning and Retention after Ablation of Inferotemporal Cortex in Dogs

By

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INTRODUCTION

In recent years, considerable theoretical interest has been concentrated on the temporal lobe. Effects of bilateral ablation of temporal lobes on general behavioral changes in monkeys[21], cats[2] and rats[20] have been reported. The inferotemporal lobe syndrome by Klüver and Bucy[9] in the rhesus monkeys is well-known and has frequently been cited.

More recently, studies on fractionized lesions in the temporal lobe of monkeys indicated that bitemporal lesion restricted to the surface on the inferior convexity of the temporal lobes was sufficient to produce visual deficits, but caused no apparent changes in affective behaviors (Chow[3], Mishkin[10]), while anteromedial temporal lesion caused affective changes rather than cognitive ones (Pribram & Bagshaw[18]).

The visual disturbances produced by the bitemporal removal were attributed to the impairments of the so-called "higher visual center" (Chow[4], Mishkin & Pribram[12], Pribram & Mishkin[19]) or the impairments of the primary visual process (Mishkin & Hall[11], Pasik *et al.*[16]), but the nature of these deficits and underlying neural mechanisms are yet to be elucidated. In order to shed further light on this problem, it was attempted in the present experiments to investigate the effects of such ablation on the dog's performance capacity by determining thresholds of visual discrimination in which a modified method of a learning sets procedure by Harlow[6] was used as a behavioral index of the intellectual performance capacity. It was investigated to what extent the learning capacity was impaired, and how the retention of what had previously been learned was after bilateral ablation of the inferotemporal lobes. In these experiments tests of different difficulties were used, for it seemed possible that the effects of ablation might be different depending upon the degree of difficulty.

The present experiments seem to provide further evidence that the bitemporal lesion produces the impairments of the so-called "higher visual center"

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rather than the primary sensory deficits.

METHOD

Subjects: Fifteen naive adult dogs were used. They had never been subjected to any kinds of conditioning before the present experiments.

Surgical procedures: After completion of the initial training, all subjects (S_S) received a one-stage bilateral operation. S_S were anesthetized with morphine (10 mg/kg) administered intramuscularly and with nembutal (20 mg/kg) administered intravenously. With aseptic technique, polus temporalis and gyrus temporalis inferior were symmetrically removed by subpial aspiration with use of a small gauge sucker (T-dog). In a control experiment, gyrus ectosylvius was bilaterally absorbed with the same surgical procedures as above (E-dog). The amount of ablated area in the E-dog was the same as in the T-dog. Some of T-dogs such as Dogs A, E and K were again subjected to ablation of the ectosylvian gyrus to make a control experiment, after all the experimental tests for the T-dog had been finished (T-E-dog). On the other hand, some of E-dogs received an additional removal of the inferotemporal lobes (E-T-dog). Between the first and second operations, there was an interval of one to three months.

Apparatus and training procedures: Experiments were performed in a soundproof room dimly illuminated by a 20-W lamp. *S*s were gently restrained in a hammock, but could freely move their four limbs. The learning of the discrimination problem was established on the basis of instrumental conditioned avoidance responses. *S*s learned to respond with a flexion of the leg to a positive conditioned stimulus (pos. CS), and not to respond to a negative CS. Both pos. and neg. CSs were presented for five seconds and the termination of pos. CS was overlapped with an unconditioned stimulus (US) lasting for two sec. Rhythmic electroshocks of 20 to 40V were used as the US, which was strong enough to elicit a flexion of the leg. One of stimulating electrodes was a needle inserted into the left hind leg and the other was a tin plate on which the leg was placed. Thus, the US could be avoided by withdrawal of the leg in response to pos. CS. Two kinds of the problems, "α" and "β", were set in the present experiments. The problem α was more difficult and the stimuli in it consisted of flicker frequency (FF) of 7.5 cps as pos. CS and that of 2.5 cps as neg. CS. In the easier problem β, FF of 10 cps as pos. CS and that of one cps as neg. CS were paired. Learning was said to have occurred when the level was attained at which more than 70 per cent correct responses were obtained to both pos. and neg. trials respectively over five consecutive days. If such a level was not attained within 1000 trials, no further trial was made. One session was run each day, consisting usually of 20 trials and balancing for pos. and neg. CSs. In each training session, pos. and neg. CSs were given in an entirely random order at intervals of 30 to 120 sec.,

in order to avoid temporal conditioning. Pre- and postoperative schedules contained overtraining[7], determination of the threshold of discrimination and retention tests in the pre- and postoperative periods in addition to initial training or retraining. Two separated experiments were conducted. In the first experiment, retention after a bitemporal lesion was investigated, and in the second, initial learning was studied in operated dogs. In the Experiment I, both problems α and β were run. In the Experiment II, the problem α was not run because it seemed to be too difficult; even for T-dogs trained initially with the problem β it was difficult to meet the criterion within the specified number of 1000 trials. After surgical intervention and five to seven days' recovery period, the postoperative relearning and the postoperative initial learning were made in the same way as mentioned above. Before initiation of the formal training, all *S*s were familialized with the experimental situations, but any kinds of preconditioning were not performed. Flickering light (FL) was produced by means of an electric stroboscope with a Xenon discharge tube, driven by a pulse generator.

Threshold of visual discrimination: After all the discrimination tests with the special pair of FFs (initial test) were finished, the capacity to discriminate between various FFs was tested at an interval of several days, paralleling with the overtraining course of the initial test. As the standard or comparison stimulus, the FF of 7.5 cps in the dogs trained with the problem α or that of 10 cps in the group trained with the problem β was used. These stimuli were always pos. CS. The standard stimulus was paired with one of the lower frequencies such as 2.5, 3.0, 3.5 cps, etc. in the dogs which learned the problem α or 1.0, 1.5, 2.0, etc. in the dogs which learned the problem β, as the variable stimulus. The variable stimuli were always negative in each pair and their frequency was raised in step of 0.5, starting from 2.5 or 1.0 cps until *S*s could no longer discriminate. Such tests were always preceded by the initial test referred to above. For each pair of FFs about 20 trials were made, balancing for pos. and neg. stimuli and testing in the same way as in the initial test. Several trials with the initial test problem were sometimes inserted to be sure that the dog's behavior to the test remained unchanged. Similar experiments were also performed after operation at various stages of relearning. When the operated dog failed in the initial test, the dog was subjected to easier tests, increasing the difference in frequency where the higher FF was fixed, but the lower neg. CS was decreased in frequency.

Informal observation of behavior: By two experimentors individually, detailed protocols were prepared concerning pre- and postoperative behavioral changes in the experimental situation and/or in the home house. Observation was made of spatial orientation, motor status, orality, sexuality, tameness or ferocity, maternal behavior, food intake, social dominance, etc.

RESULTS

1. Limitation of lesions

After the experiments were finished, the animals were sacrificed with intravenous nembutal injection. The brains were perfused with 10 per cent formalin, embeded in celloidin and then serially sectioned at 40μ thickness. Frontal sections, stained with carbol-thionin, were examined macroscopically to identify lesions and also examined microscopically. Figs. 1 and 2 show the position and extent of the lesions in the Experiment I, together with representative frontal sections. Fig. 3 shows those of Experiment II. In these figures, ventral and lateral views are shown and lesions are indicated by black areas.

Generally speaking, the ablation conformed well to the experimental plan

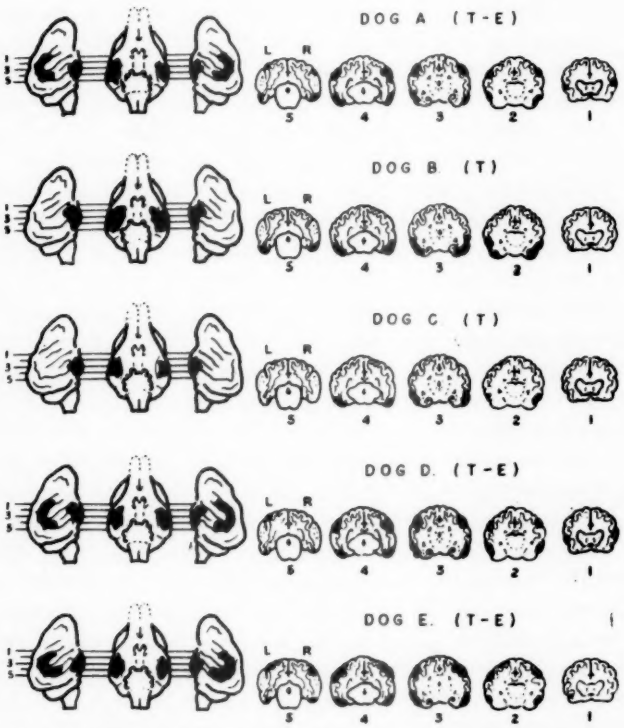

Fig. 1. Reconstructions of lesion in animals subjected to Experiment I on retention. Five representative frontal sections are shown to the right of lateral and ventral views of brain. Numbers for sections correspond to those given in lateral views.

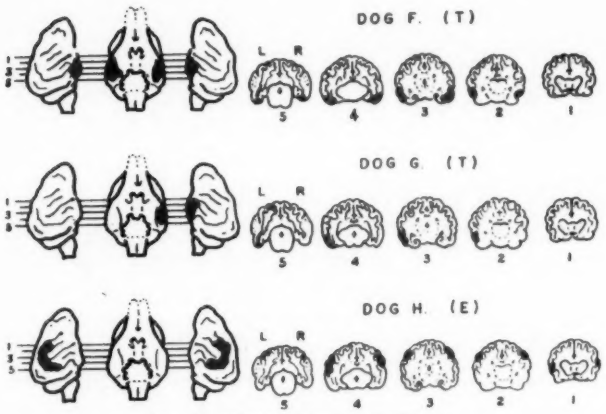

Fig. 2. Explanation as in Fig. 1.

in which the ablation of polus temporalis and gyrus temporalis inferior was aimed at. However, some relatively minor deviations were noted ; an inferior portion of gyrus ectosylvius posterior or/and gyrus Sylvii posterior was more or less damaged in all cases, and the further lateral side of pyriformis was slightly invaded in some cases. Amygdala and hippocampus were entirely intact except for Dogs B, F and I. Dog B showed considerable deviation from the intended removal. In this dog, amygdala was largely removed in addition to the intended areas ; n. basalis amygdalae parvicellularis and an inferior portion of n. lateralis amygdalae were bilaterally removed. N. cortalis amygdalae and n. basalis amygdalae magnocellularis were also invaded a little in the right hemisphere. An inferior portion of the right ventral hippocampus was also invaded slightly. The right hemisphere received more damage than the left did. Amygdala was almost safe in Dogs F and J, although some of the lateral half of pyriformis was invaded, as shown in Figs. 2 and 3. Dog F received a lesion in an inferior portion of the ventral hippocampus in the right hemisphere. Dog I received slight destruction of an inferior portion of the right lateral amygdaloid nucleus. The ablation in Dog C was not sufficient ; sparing of a posterior portion of the bilateral inferior temporal gyri and sparing of medial side of the left temporal pole were observed.

In the control group, the removal was also in agreement with the intended areas of gyrus ectosylvius and an upper portion of gyri ectosylvius posterior and anterior, which belong to the auditory area. The amount of ablated area was the same as that of the inferotemporal lobe. Small deviation was also seen in the left hemisphere of Dog M ; a little sparing of the ectosylvian gyrus and an invasion of the anterior Sylvian gyrus were seen.

The terminology used here to designate gyri, sulci as well as specific areas

Fig. 3. Reconstructions of lesion in animals subjected to Experiment II on acquisition. Explanation as in Fig. 1.

was taken from "A Stereotaxic Atlas of the Dog's Brain" by Lim, Liu and Moffitt[9].

2. Informal observation of behavior

a) General behaviors

Although detailed prótocols were prepared, the T-dogs did not show any remarkable behavioral changes after operation. Neither ataxic symptoms nor gross visual disturbances could be observed. But in some cases temporary changes lasting for several days were observed; they represented an incomplete form of the Klüver-Bucy syndrome which consisted of visual agnosia, oral tendency, hypermetamorphosis and changes in emotional, sexual and dietary behaviors. The E-dogs did not show any kinds of the above-mentioned behavioral changes, but displayed some behaviors like sniffing.

An attractive episode was provided by Dog B from which a large part of amygdala in addition to the inferotemporal surface was bilaterally removed.

Preoperatively, this dog had been pregnant and reared a litter of puppies successfully. After operation, the dog never wanted to breed hers and appeared to feel annoyed by them. This dog also displayed homosexuality and tended to get excited easily by trifles. This episode continued until this dog was sacrificed three months later.

b) Spatial orientation

The change in spatial orientation was the most marked and common one observed after operation. Before operation, Ss would run back straight to their house when released from the test room. The T-dogs were different from normal in this behavior ; they wandered about aimlessly and would not go home. They appeared, in general, less active and indifferent to surroundings, but they were capable of responding positively to anykinds of CSs with a flexion of the leg and making discrimination between pos. and neg. CSs to some extent.

On the contrary, the control dogs were not affected in orientation and could respond to experimentors after operation in the same manner as before operation.

3. Experiment I

a) Experimental group

It was attempted in this experiment to investigate retention and reacquisition of the visual performance after bilateral ablation of the inferotemporal cortex with special reference to the degree of difficulty of tests. Before operation, the number of trials necessary for attaining the criterion of learning was from 360 to 440 with the problem β and more than 600 with the problem α. Therefore, the problem α was more difficult than the problem β. Examples of learning curves with the problem α are shown in Fig. 4, in which percentage frequencies of CRs are plotted as ordinates against days of training as abscissae. Open circles denote positive responses to pos. CS and solid ones refer to positive responses to neg. CS. The greater the difference of both curves, the better discrimination. It was so defined that discrimination was successful when the positive responses to pos. CS was higher in occurrence than 70 per cent, while the positive responses to neg. CS was lower than 30 per cent. Such a state was preoperatively attained after 30 days during which 600 trials were made. After operation (lower diagram), learning capacity was so much affected that the criterion could not be reached even after 65 days or 1140 trials. In Fig. 5, learning curves of another dog (Dog F) which was trained with the easier problem β are shown. In this case, 18 days or 360 trials were enough to reach the criterion before operation, and 13 days or 260 trials were required to relearn after operation.

Problem α : Although eight dogs were conducted with the problem α prior to operation, only two of them succeeded in learning. Dog A got the criterion of the problem α after 900 trials preoperatively (4th column in Table I) and

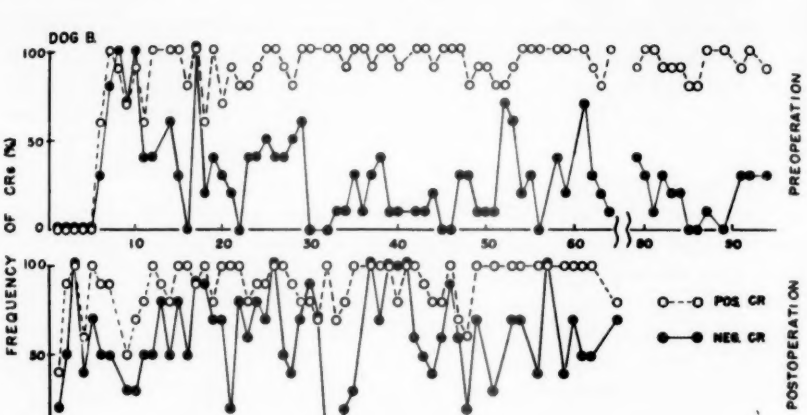

Fig. 4. Pre-(upper) and post- (lower) operative learning curves determined with a more difficult discrimination task α (Dog B). Open circles denote percentage frequencies of positive conditioned responses to positive conditioned stimuli, and filled circles are those to negative conditioned stimuli. Asterisk (*) denotes "educational effect" described in text.

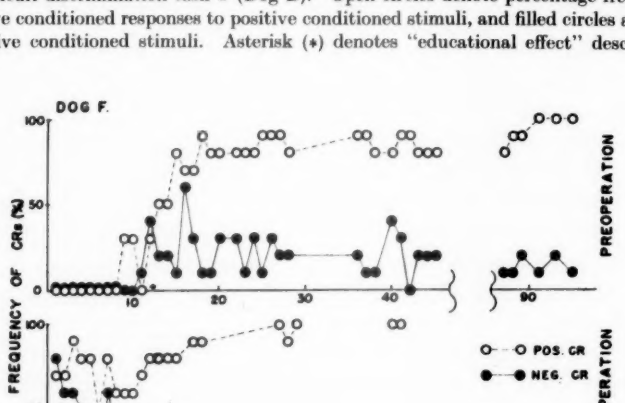

Fig. 5. Pre- and postoperative learning curves determined with an easier discrimination task β (Dog F). Explanation as in Fig. 4.

could relearn it within 960 trials postoperatively (7th column in Table I). In view of the fact that some retention was observed in this dog immediately after operation, the postoperative scores 960, which were larger than the preoperative

ones 900, indicated that the learning capacity of this dog was affected by the operation. Another Dog B, which counted the number of 600 trials until the preoperative criterion was attained, failed to relearn the problem α after operation even with more than 1140 trials, twice the preoperative learning scores. Postoperatively, Dog B could easily performed the easier problem β with which this dog had not been trained initially, whereas this dog could not reach the criterion in learning of the problem α. Strange to say, a few trials of presentation of the easier problem β prior to that of the problem α made this dog reach the criterion in learning of the problem α. It seemed as if success in an easier task increased the discrimination capacity, though temporarily. The effect was called an "educational effect" in this paper. As shown in the postoperative learning curves of Fig. 4 (lower diagram), the learning was once established on the 32nd and 33rd days and this was due to the educational effect. Dog C could not meet the criterion within 1200 trials preoperatively, but discrimination as tested by the problem α seemed to be better than mere chance. After operation, discrimination capacity was entirely lost in such a manner that the dog showed only positive responses to all kinds of stimuli (stage of generalization).

Problem β: Five dogs were subjected to tests with the problem β and could easily learn them after 360 to 440 trials. The complete retention of the performance could not be seen right after operation, but all *S*s could relearn the

TABLE I. Learning Scores of Visual Discrimination (Experimental Group of Experiment I)

Animal	Problem	Preoperative				Postoperative		
		Trials necessary to cause first CR	Initial learning	Retention	Threshold (cps of neg. CR)	Relearning	Retention	Threshold (cps of neg. CS)
Dog A	α	140	880	0	4.0	940	0	1.0
Dog B	α	100	580	20	4.0	1140*	—	1.0
Dog C	α	140	1200*	—	—	1020*	—	—
Dog D	α	180	840*					
	↓		↓					
	β		80	0	2.5	360	0	0.5
Dog E	β	180	420	0	4.0	60	0	3.0
Dog F	β	160	340	0	3.0	240	0	0.5
Dog G	α	220	900*					
	↓		↓					
	β		0	0	2.5	0	0	3.0
Dog L	β	120	300	0	3.5	Died on the way of relearning		

Note: Scores represented in above table are trials required to attain criterion. Scores of threshold presented here are last preoperative values and first postoperative ones. Asterisk (*) denotes failure to acquire the habits within the specified number of training trials. Arrow indicates that the imposed problem "α" was changed into "β" after failure of learning on "α". Dashed line represents that dog was not tested at that stage.

problem rather rapidly as shown in Table I (columns 4 and 7). The so rapid relearning in Dog E, for which only 80 trials were sufficient, was probably due to the circumstance that preoperative overtraining[7, 13] was more extensive in this dog than did in others. Fig. 6 shows how easy the problem β was to relearn

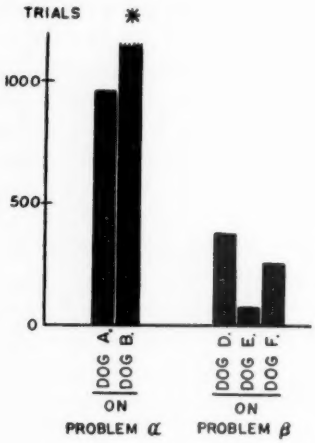

Fig. 6. Postoperative relearning scores (Experiment I on retention). Number of trials required to reach criterion is greater for group trained with more difficult problem α than for that with easier problem β. Asterisk (*) denotes failure in reaching criterion.

postoperatively in comparison with the problem α. Dog L, which had received operation in both ectosylvian gyri, showed a complete retention of the preoperative learning after operation. In the same dog, ablation of both inferotemporal lobes was carried out one and a half months after the first operation. After this second operation, a great deficit in visual performance was found, although it was impossible owing to the death of the dog to determine whether or not this dog would be able to relearn the problem in the long run. Dog G, which had received a left unilateral lesion in the inferotemporal lobe, showed a complete retention.

It may be concluded from these findings that loss in learned visual performance was greater with the more difficult problem α than with the easier problem β.

b) *Control group*

Bilateral ablation of ectosylvian gyri: For control to the effects of bilateral ablation of the inferotemporal lobes upon the deficits of visual discrimination, gyrus ectosylvius which belongs to an auditory area was bilaterally ablated. The amount of ablated area was made almost the same as that of the inferotemporal lobe as shown in Figs. 1, 2 and 3. The results obtained are represented in Table II. The discrimination learned by preoperative learning was well-retained. The threshold of discrimination was found to be the same as before opera-

TABLE II. Learing Scores of Visual Discrimination (Operated Control Group of Experiment I)

Animal	Problem	Preoperative		Postoperative		
		Retention	Threshold (cps of neg. CR)	Relearning	Retention	Threshold (cps of neg. CR)
Dog A	α	0	4.0	20	0	4.0
Dog E	α	0	4.5	40	0	4.0
Dog K	α	0	3.5	Died after surgery		
Dog H	β	20	2.0	0	0	2.0
Dog D	β	0	2.0	0	0	2.0
Dog G	β	0	2.5	0	—	3.0

Note : Refer to TABLE I.

tion. The number of trials in preoperative overtraining in this series was made far less than that of the T-dogs in order to control the effects of overtraining[7].

Unilateral ablation of inferotemporal lobe: In Dog G which was subjected to a unilateral ablation of the left inferotemporal lobe, the complete retention of the performance was observed after operation. The threshold of the visual discrimination of FFs was found improved a little in this dog. No affective changes were observed, either. These findings might indicate that the unilateral resection of the infeortemporal lobe did not affect the cognitive and affective behaviors.

Retention tests: It took usually a week for recovery from operation. The reduction in performance capacity after operation may be due to a normal process of forgetting. In order to answer this question a series of retention tests was performed preoperatively as well as postoperatively. The results obtained are summarized in Table I (columns 5 and 8). As can be seen in this Table, the retention was complete before operation as well as after operation. It is to be noted that the retention test after operation was made after relearning.

4. Experiment II

In Experiment I all dogs experienced learning preoperatively. In the Experiment II, no special procedure of learning was performed before operation to examine the effects of operation upon the initial postoperative learning without influence of any preoperatively acquired memory. The rate of acquisition could be expressed by two indicators. The one was the number of trials required from commencement of training to the first CR to any kinds of flickering lights, and the second was that required for completion of learning. The former indicator refers to the number of trials which will be needed until the dog becomes aware of the relation between CS and US. Three T-dogs and two E-dogs were used in this experiment.

The results are summarized in Fig. 7. As shown in the left diagram of this figure, the first indicator, i.e., the number of trials needed for the first CR was

much greater for the T-dogs than for normal or non-operated dogs (N-dogs). The operated control dogs or E-dogs were the same as the normal ones in this behavior. The second indicator, i.e., trials-score needed to reach the criterion of learning was also much higher in the T-dogs than in the normal dogs and the E-dogs (see the right diagram in Fig. 7).

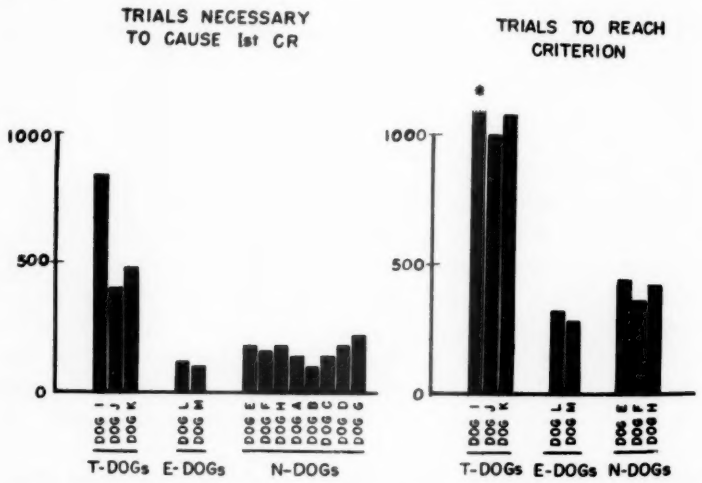

Fig. 7. Initial postoperative learning scores (Experiment II on acquisition). Columns in left figure denote trials required to cause first conditioned response and those in right indicate trials to reach criterion in learning. Temporal-operated dog: T-dog; Ectosylvius-operated dog: E-dog; Non-operated or normal dog: N-dog. Asterisk(*) denotes failure in reaching criterion.

5. Threshold of visual discrimination or differential threshold (DT).

In order to investigate the manner of the pre- and postoperative performance capacity, the DT was determined in all dogs. The procedure used here was a modification of learning sets — learning-how-to-learn — procedure by Harlow[6]. A graded series of FFs was presented as neg. CSs, while the frequency of a pos. CS was fixed at a certain higher value.

When the difference in frequencies of pos. and neg. CSs was large enough, the dog showed a positive response to the pos. CS, but no response to the neg. one. As the difference decreased, the dog showed a positive response to the neg. CS more and more frequently. It was so defined that a differential threshold was attained when positive responses occurred to neg. CSs in 30 per cent of all trials with neg. CSs.

The changes in DT at pre- and postoperative stages are shown in Fig. 8. In this figure, the highest frequencies of the neg. CSs discriminated from the pos. CS were plotted as ordinates against the days of training as abscissae. Im-

pairment and improvement of discrimination capacity are indicated by a fall and a rise of curve respectively. Dogs A and B were trained with the problem α, difficult one. The other dogs D, F and E were trained with the problem β, easier one. The highest possible frequency of neg. CS was 2.5–4.0 cps at the preoperative stage (column 6 in Table I), but was found to be much lowered to 0.5–1.0 cps about a week after bilateral ablation of the inferotemporal lobes (column 9 in Table I). This finding indicates that the discrimination capacity has strongly been affected by the operation. However, all dogs except Dog B showed complete recovery concerning the discrimination capacity after further training at the postoperative stage. After operation, the discrimination capacity was not so strongly reduced in Dog E as in the other dogs. This fact may be due to the circumstance that more intensive overtraining was carried out preoperatively in Dog E than did in the others[7]. Dog B, the exceptional case in which no recovery was seen, did not succeed in postoperative tests with the problem α which was solved with success before operation, although this dog could make discrimination on the easier ones.

It is without saying that the postoperative discrimination capacity in the control dogs remained at the same level as at that before operation.

DISCUSSION

It has been shown above that marked impairments in visual discrimination of FFs were produced by bilateral ablation of the neocorticies on the inferior convexity of the temporal lobes, i.e., polus temporalis and gyrus temporalis inferior. Deficits in visual discrimination were revealed by three kinds of experiments on retention, acquisition and threshold of discrimination. Testing and training procedures and animal species used here were entirely different from those in the previous studies[12,19]. Nevertheless, the results obtained in the present experiments are similar to those obtained from monkeys.

As to the mechanism of the impairments, there have been controversies; some investigators[4,12,19] postulated damages in the so-called "higher visual center", while others[11,16] assumed the disturbances of the primary visual process. The latter hypothesis seems, however, unlikely for the following reasons. For example, Dog B could never solve the more difficult problem α without several preliminary trials with the easier problem β. It would be difficult to account for such an educational effect in terms of mere primary sensory deficits. Furthermore, it was directly revealed that the capacity to form learning sets which may indicate a behavioral degree of the intellectual performance was markedly impaired after inferotemporal operation. As has been shown above, the number of trials required to evoke the first CR was greater in the T-dogs than in the control dogs. This fact indicates that the mechanism for conditioning has been affected by operation. It is apparent that the central mechanism for conditioning

is not at all restricted to the primary sensory mechanism. Judging from the behavior of the experimental animals, no gross defect was found in the visual

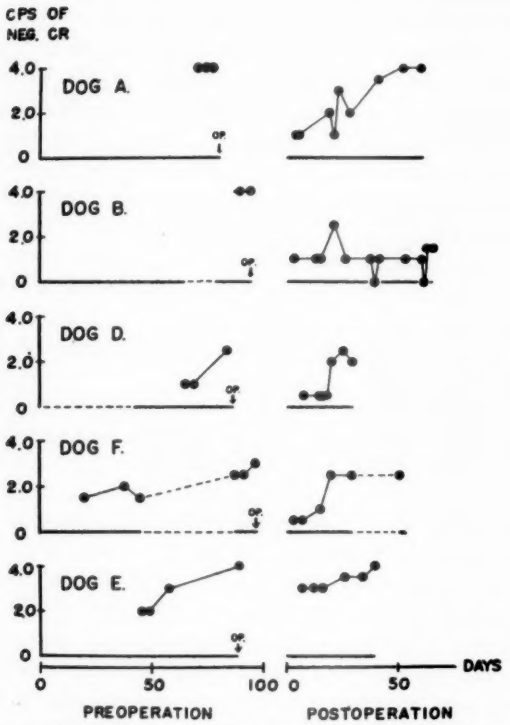

Fig. 8. Discrimination capacity at various pre-(left) and post-(right) operative stages. The higher flicker frequency serving as a pos. CS was fixed, and the frequency of neg. CS was varied. The highest frequency of neg. CS discriminated from pos. CS was plotted against days of training. Dashed lines of abscissae in Dogs B and F denote period of quiescense of training for retention tests. Dashed line in Dog D indicates period of training with problem a, which this dog could not learn.

field. It has often been reported that little retrograde degeneration of the lateral geniculate body was observed in the temporal-operated animals, in addition to the absence of defects either in visual field or in acuity[1,12]. Thus, the impairments mentioned above may be considered to be specific for the ablated inferotemporal areas, and this interpretation receives support from the fact that no such impairments occurred in the control E-dogs. There is some evidence that an interruption of the thalamo-cortical relation or ablation of the prestriate cortex affected little visual discrimination (Blum *et al.*[1], Chow[5]). Even if these

systems had been damaged in our experiments, it would be difficult to interpret the impairments under consideration as due to the damages in these systems. It was reported that the T-animals showed complete retention of conditioning behavior, so far as simple conditioning with various visual stimuli was concerned (Chow[3], Mishkin & Hall[11], Pribram & Mishkin[19]). In the present experiments, it was represented that Dog C, which could not solve the problem α before operation, showed a complete generalization form to CSs after operation and that the retention of preoperatively learned discrimination was relatively high when the easier problem β was used. This suggests that the T-dogs might be nearly normal in simple conditioning which started before operation as in the earlier experiments. It is, however, to be noticed that initial learning started post-operatively was found very difficult, as shown above.

It is suggested in the literatures that different parts of the temporal lobe are not equipotential for visual discrimination capactity of animals. In the present experiments, polus temporalis and gyrus temporalis inferior were resected without involvement of the deep allocortical structures and such remarkable visual impairments as stated above have been obtained. The present results agree with earlier reports on monkeys suggesting that the ventrolateral surface of the temporal lobe mediates visual discrimination[3,10,12], while that no visual deficits were caused by removal of the anteromedial region of the temporal lobe or destruction of allocortical structures[18]. The question as to which is more essential to visual function, polus temporalis or gyrus temporalis inferior, is not yet settled (Mishkin[10], Pinto Hamuy *et al.*[17]).

Whether or not the disturbances produced by the inferotemporal ablation are modality-specific is also an open question (Blum *et al.*[1], Orbach *et al.*[14]).

Dog B, which received the lesion of a large part of bilateral amygdalae in addition to the intended areas, displayed remarkable changes in affection. The other dogs in which lesion did not reach the deep allocortical regions showed only cognitive changes, but displayed no affective ones. This finding seems to support the well-known theory that allo- and neo-cortical structures are concerned with affective and cognitive functions respectively (Papez[15]).

No permanent deficits could be observed in all but two dogs. Even these two dogs might show complete recovery if training had been continued beyond 1000 trials. The mechanism of recovery is not known at present.

SUMMARY

Based on instrumental conditioned avoidance responses, retention and acquisition of visual discrimination of flicker frequencies were studied in 15 dogs with surgical lesion limitted to the bilateral neocortecies of polus temporalis and gyrus temporalis inferior. In addition, the differential threshold of visual performance, i.e., the least difference of flicker frequencies to be discriminated

was determined at various pre- and postoperative stages in order to investigate the capacity to form learning sets, i.e., learn how to learn. Histological control of the ablated areas was made by a conventional method.

The results obtained are as follows:

1. The retention of the preoperatively learned discrimination was markedly reduced by bilateral ablation of the inferotemporal cortex, while it was not affected in the control animals which underwent bilateral removal of gyrus ectosylvius or unilateral lesion of the inferotemporal lobe.

2. For the inferotemporal-operated dogs, learning was further more difficult than for the control dogs.

3. Impairments in retention and acquisition were more marked, when tested with more difficult tasks.

4. The differential threshold of visual performance was remarkably impaired by ablation of the inferotemporal lobes, but it recovered to the preoperative level after a long period of training. It was, however, not affected by the control operation.

5. The inferotemporal-operated dogs showed some kind of spatial disorientation, while the control dogs did not.

6. The significance of the inferotemporal lobe for visual performance was discussed.

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Simultaneous Two-channel Frequency Analyzer Designed by the Authors

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Initiated by Grass and Gibbs[1], many kinds of EEG analyzing instrument have been designed, but it seems necessary to utilize a more simplified and practical technique in accordance with necessity for clinical application. For example, using one-channel frequency analyzer, it is impossible to make simultaneous analyses of two EEGs recorded from different areas. Especially in clinical field, we feel, however, such a necessity to compare two EEGs simultaneously each other. Meister *et al.*[2] have designed one type of the so-called simultaneous two channel frequency analyzer in recent years, and we also have just now devised an apparatus to be described below.

The block diagram of this apparatus is shown in Fig. 1.

The information is received from the power terminal of the analyzing channel of the EEG and the channel for this use is determined by channel selecter marked as "S_1". Gain control is accomplished by "G".

This apparatus has seven filter units such as "$FU_1 \sim FU_7$" and can be used in two ways: a) Simultaneous analysis conducted from two parts, which is possible by changing S_3 to "b", dividing the filters in two groups (labeled A and B), and b) analysis conducted from one point. By changing S_3 to "a", the analysis is accomplished by seven filters. And, thus, seven instantaneous outputs of the analyzer may be recorded by channels of the EEG pen recorder.

The output of each band may be integrated for a period of 10 seconds. In this case the integrated outputs are recorded by an auxiliary pen.

The pass bands of the filters are estimated as follows; δ_2: 1–2 c/s; δ_1: 2–4 c/s; θ: 4–8 c/s: α: 8–13 c/s: β_1: 13–20 c/s; β_2: 20–30 c/s and β_3: 30–60 c/s. Each frequency peculiarity of the filter for δ-, θ-, α- and β- band is illustrated in Fig. 2.

The cut off frequency of each band of these filters has the minimal dump

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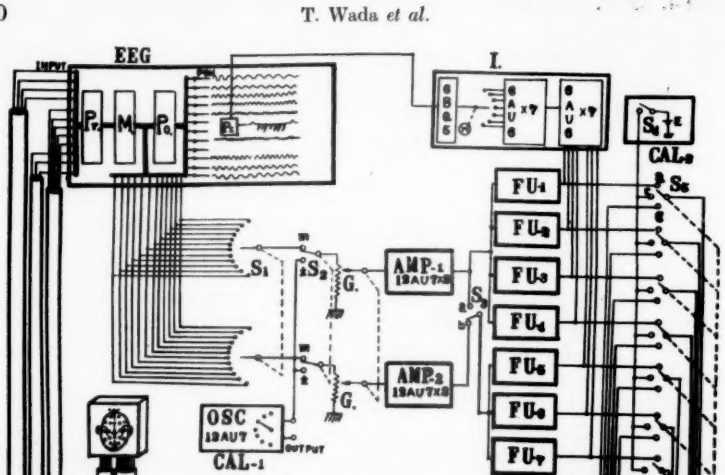

Fig. 1.
Diagram of apparatus.

EEG : Electroencephalograph
Pr : Preamplifier
M : Mainamplifier
Po : Poweramplifier
PEN : Recording pen of EEGs and instantaneous values
P_1 : Recording pen of integrated value
I : Integrator
(M) : Switch changed by electric motor
6BQ5 : Vacuumtube
6AU6 : Vacuumtube
CAL-1 : Calibrator for instantaneous value
OSC : Oscillator
CAL-2 : Calibrator for instantaneous value
S_4 : Switch for calibration
FU : Filter unit
AMP-1 & AMP-2 : Amplifier
G : Gaincontroler
S_1 : Channel-selector
S_2 : Switch m, for Analysis
t, for Calibration
S_5 : Switch a, for Analysis
c, for Calibration
e, for EEG

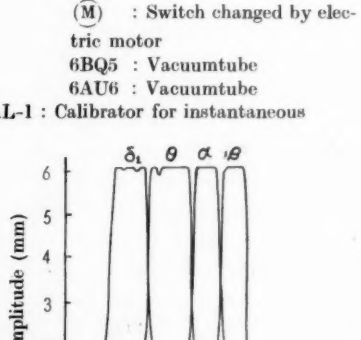

Fig. 2.
Character of pass-band of filter employed.

peculiarities 1) to eliminate the disturbance to be introduced by abrupt artificial phenomena and 2) to pass and analyze accurately the EEG wave with variable amplitude.

This analyzer has the oscillator (OSC) within circuit, through which man can test every filter unit. It is possible to generate 100 mV, a maximal output voltage, from OSC. And, by this voltage output, correlation of the instantaneous analyzed value and the integrated one is estimated. The correlation curve of the instantaneous analyzed value is made by S_4 of CAL_2. The minimal input value is 1.5 volts and 2MΩ in impedance. The maximal amplitude of the integration due to recording pen is 120 mm, and the impedance of this pen motor is 3KΩ. The band deviation of A and B channels is no greater than $3\pm10\%$ in every part.

Next, 2 samples of analyzed EEG figure are shown. Fig. 3 shows the analyzed result from the occipital region of a normal subject, in which "A" means the left side and "B" the right. The upper three traces show the instantaneous values of EEG-A and the lower three ones those of EEG-B. The curve in the middle of the figure indicates the integrated value ; the first three curves are those of δ_1-, θ- and α-band of EEG-A and the next three, those of EEG-B. The amplitude of α-band is remarkably decreased by photic stimulation in both hemispheres.

Fig. 4 is an example of another case. At a glance, a significant difference between both hemispheres may be recognized.

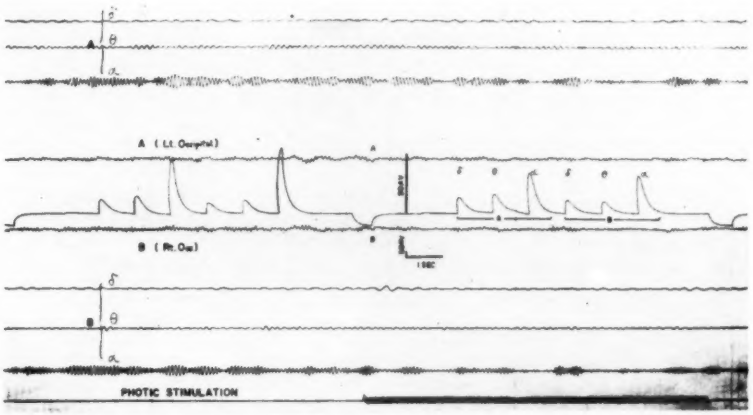

Fig. 3. Sample of analyzed EEG ; normal adult. While the integrated value in the first epoch (10 seconds) showed 46 μV in the α-band of A (the upper) EEG and 56 μV in that of B (the lower), in the next epoch in which photic stimulation with 10c/s was applied, that of the former decreased to 34 μV and the latter, to 31 μV.

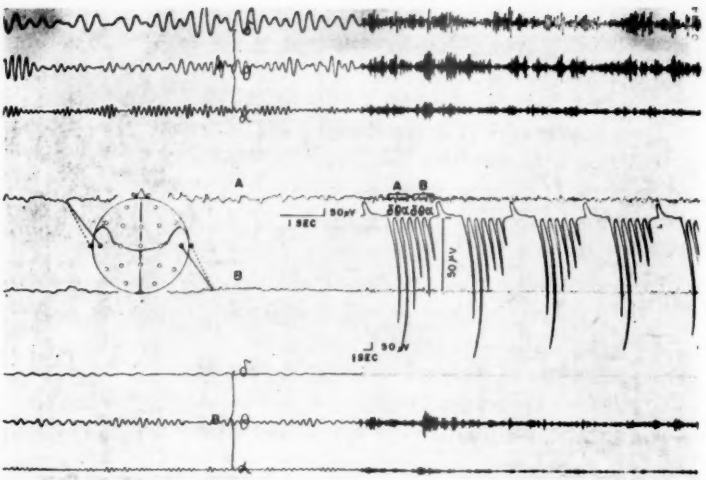

Fig. 4. Sample of analyzed EEG ; a case of temporal lobe epilepsy with asymmetry in the temporal region. In each band, EEG-A (the left mid-temporal area) shows a remarkable higher value than in EEG-B (the opposite side). Thus quantitative comparison was made much easier.

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Enzymatic Anomaly of Erythrocytes in Congenital Nonspherocytic Hemolytic Anemia

(The Second Report)

By

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In a previous paper,[1] the author reported an enzymatic anomaly of erythrocytes, consisting of a low content of reduced glutathione (GSH), an abnormality in the reduced glutathione stability test, and a low activity of glucose-6-phosphate dehydrogenase (G6PD), in a pedigree of congenital nonspherocytic hemolytic anemia. Recently the same enzymatic anomaly of erythrocytes was demonstrated in the family of another pedigree. In this paper, the enzymological studies in both pedigrees were described.

Case I. A 40-day-old girl was admitted to our Hospital because of severe, prolonged jaundice from the 4th day of life. The family history revealed that an elder brother and a maternal first cousin died of jaundice in early infancy

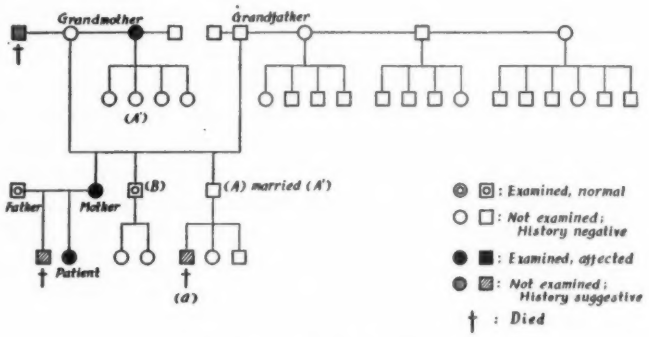

Fig. 1. Pedigree of case I

(cf. Fig. 1). There was no history of jaundice or anemia in the relatives of the father's side. The parents were not consanguineous but appeared to be healthy.

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An uneventful full-term pregnancy and spontaneous delivery were noted. Physical examination on admission showed a well-developed infant except remarkable jaundice and hepatosplenomegaly. The liver with smooth surface was palpable 4 cm below the right costal margin and the spleen was palpable 1.5 cm below the left costal margin. Otherwise, no abnormal findings were present.

Case II. A 14-year-old boy was admitted to our Hospital because of unusual hemolytic anemia. Jaundice and anemia occurred soon after birth and had ever continued with fluctuation of severity from time to time. The family history revealed a high incidence of anemia in the relatives of the mother's side but none in the father's side (cf. Fig. 2). The parents were not consanguineous

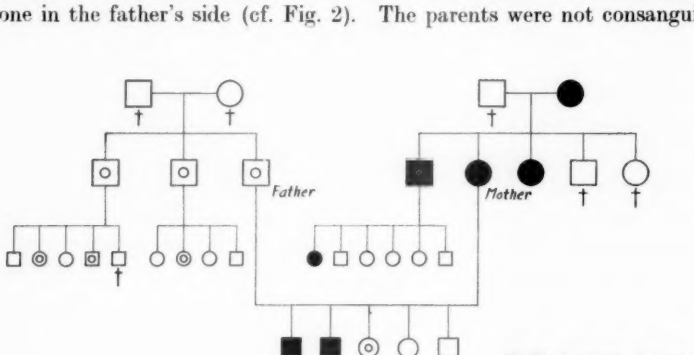

Fig. 2. Pedigree of case II

but appeared to be healthy. Physical examination on admission showed a moderately nourished boy with subicteric and pale skin. The liver was not palpable but the spleen was palpable 2 cm below the left costal margin. Otherwise, no abnormal findings were present.

Laboratory examinations: No blood-group incompatibility between the patient and the mother could be demonstrated in either case. The Coomb tests, both direct and indirect, and Wassermann's reaction were negative in both cases. Hematological examinations of both patients (cf. Table I) showed a mild anemia and reticulocytosis with the absence of spherocytes, of normoblasts or of any other morphologically abnormal erythrocytes. Elevations of icterus index and of serum bilirubin, especially of indirect fraction, were remarkable and the Hijmans van den Bergh reaction was negative for direct and markedly positive for indirect in both patients (cf. Table II). Excretion of urinary and fecal urobilinogen also was increased. The liver function tests were within normal

TABLE I. Hematological Examinations.

		Case I				Case II				
		Patient	Mother	Father	Uncle (B)	Patient	Mother	Father	Brother	Sister
R.B.C.	M/mm³	3.8	3.5	4.9	4.9	3.0	3.8	5.1	3.7	4.2
Hemoglobin	g/100ml	14.6	12.0	16.0	15.5	9.7	11.2	14.8	12.1	13.2
W.B.C.	/mm³	9400	4800	6200	6800	6800	7850	7600	8200	8650
Hematocrit	%	36	35.5	44	42.5	28	35.5	48	35.5	38
Color index		1.25	1.12	1.09	1.06	1.08	0.98	0.97	1.09	1.05
Volume index		1.03	1.11	1.00	0.97	1.04	1.04	1.05	1.06	1.00
M.C.V.	μ^3	93	100	90	88	93	93	94	96	90
M.C.H.	$\gamma\gamma$	37.7	33.8	32.6	32.0	32.3	29.5	29.0	32.7	31.4
M.C.H.C.	%	40.5	33.8	36.4	36.5	34.6	31.6	30.9	34.1	34.8
M.C.D.	μ	7.31	7.34	7.57		7.60	7.70	7.75		
M.C.T.	μ	2.22	2.37	2.00		2.05	2.00	2.00		
Spherocyte index		0.30	0.33	0.26		0.27	0.26	0.26		
W.B.C. Differential: Lymphocytes	%	74.0	44.0	32.0	38.0	33.0	14.5	36.0		
W.B.C. Differential: Neutrophils	%	20.0	44.5	62.0	56.0	61.5	77.5	55.0		
W.B.C. Differential: Monocytes	%	3.5	7.0	4.0	5.0	0.5	7.0	7.0		
W.B.C. Differential: Eosinophils	%	2.0	3.0	1.5	1.0	4.5	1.0	1.5		
W.B.C. Differential: Basophils	%	0.5	1.5	0.5	0	0.5	0	0.5		
Reticulocytes	‰	42	20	8	7	120	31	14	35	10
Platelets	/mm³	235,000				189,000				
Bleeding time	min.	1 1/2				2 1/2				
Coagulation time	min.	5-8 1/2				6-13				
Osmotic fragility	%	0.42-0.32				0.42-0.34				

limits (cf. Table II). E.K.G. and X-ray examination revealed no evidence of abnormalities in both patients. An evidence of hemolytic anemia in a milder degree was disclosed by examinations in their mothers who seemed to be healthy in appearance (cf. Tables I and II). Basing upon the above-mentioned results, the diagnosis of congenital nonspherocytic hemolytic anemia was made for both cases.

GSH content in erythrocytes and the GSH stability test (cf. Table III) The determination of GSH content in erythrocytes and the GSH stability test were carried out as described in a previous paper of mine.[1] The erythrocytes of both patients had a low level of GSH as compared with that of controls. As to the GSH stability test, GSH in erythrocytes from the patients showed a remarkable drop after incubation with acetylphenylhydrazine, while GSH in erythrocytes from controls remained almost unchanged after the incubation. The erythrocytes from the mothers showed a relatively low content of GSH as well as diminished stability of GSH in a milder degree. On the other hand,

TABLE II. Biochemical Examinations.

		Case I				Case II				
		Patient	Mother	Father	Uncle (B)	Patient	Mother	Father	Brother	Sister
Serum	Icterus index	94	12.5	8.0	7.0	23	13.5	6.0	16.0	5.0
	Hijmans van den Bergh direct	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
	Hijmans van den Bergh indirect	(±)	(+)	(−)	(−)	(±)	(+)	(−)	(+)	(−)
	Bilirubin mg/dl total	10.0	1.0	0.6	0.5	3.7	1.2	0.5	1.4	0.4
	Bilirubin mg/dl indirect	7.9	0.7	0.1	0.1	3.2	1.0	0.2	1.1	0.1
Liver function tests	Thymol turbidity	1.0	0.8	1.3	1.8	3.2	1.2	1.5	1.3	2.0
	Takada's reaction	(−)				(−)				
	Cobalt reaction	R_3				R_6				
	B.P.S. 30 min. %	3.0				2.5				
	Alkaline phosphatase B.U.	5.0				4.6				
Urine	Urobilinogen (Lepehne's method)	16times (+)	(±)	(−)	(−)	32times (+)	2times (+)	(−)	4times (+)	(−)
	Bilirubin	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
	Sugar	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
	Protein	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)
	Urobilinogen/day	>100mg				>600mg				
Feces	Parasite	(−)				(−)				
	Occult blood	(−)				(−)				

the erythrocytes from the fathers showed normal values both in GSH content and in the GSH stability test.

G6PD activity in erythrocytes (cf. Fig. 3)

The activity of G6PD in erythrocytes was assayed as described in a previous paper[1] of mine. The erythrocytes from both patients showed a very low activity as compared with those from controls. The erythrocytes from the mothers showed a moderate degree of deficiency of G6PD activity, while those from the fathers showed normal activity.

Among those family members tested in each family, there was a definite correlationship between the severity of hemolytic anemia and metabolic abnormalities in erythrocytes—low activity of G6PD, low content of GSH and GSH instability. Further experiments are related in the DISCUSSION.

DISCUSSION

The metabolic anomaly of erythrocytes, consisting of a low content of GSH, GSH instability and a low activity of G6PD, was for the first time demonstrated by Beutler *et al.*[2-7] in drug-induced hemolytic anemia. The present author was able to demonstrate the same lesion in erythrocytes from two families of congenital nonspherocytic hemolytic anemia, although my own patients had hemolytic

TABLE III. GSH Content in Erythrocytes and GSH Stability Test.

		GSH (mg/100cc RBC)	GSH (mg/100cc RBC) after 2 hours of incubation with APH*	Rate of decrease of GSH during incubation
Control	Adults (5 cases)	74.5 ± 4.8	69.0 ± 4.6	7.4% ± 3.1
	Infants (5 cases) 1 - 2 months of age	88.3 ± 6.1	81.9 ± 4.4	7.0% ± 4.0
	Children (5 cases) 10 - 15 years of age	74.9 ± 7.6	68.1 ± 5.4	8.8% ± 6.3
Case I	Patient	43.6	12.8	70.6%
	Mother	59.5	40.8	31.5%
	Father	72.0	66.3	8.0%
	Uncle (B)	67.5	62.5	7.5%
Case II	Patient	59.0	28.5	51.7%
	Mother	68.5	45.0	34.3%
	Father	79.0	73.0	7.6%
	Brother	67.5	50.5	2.25%
	Sister	79.0	70.5	10.8%

* Acetylphenylhydrazine

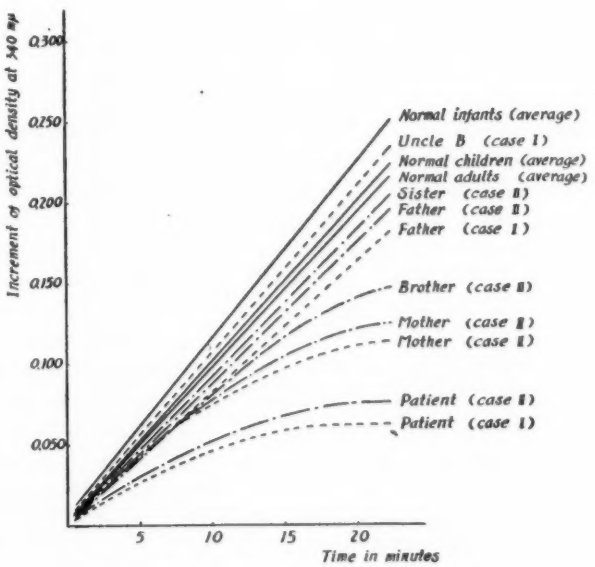

Fig. 3. G6PD activity in erythrocytes

anemia soon after birth without exposure to any drug. Now Beutler's syndrome and that in my own cases—are they of the same clinical entity? The difference seems to lie in the inducing agent. In the former the agent was certain drugs and in my own cases such an apparent agent was lacking. Can this be indeed an essential difference between the two? Now I tried to solve this problem by a

Table IV. Influence by Administration of Domian and Phenacetin to the Patient of Case II.

Date	Nov. 6	7	8	9	10	11	12	13
Dosage	← Domian 4g/day for 2 days →				← Phenacetin lg/day for days →			
R.B.C. M/mm³	3.05		2.20				2.90	
Hemoglobin g/dl	9.7		7.5				10.5	
Icterus index	23		41				18	
Serum bilirubin (mg/dl) { total	3.7		5.0				3.2	
Serum bilirubin (mg/dl) { indirect	3.2		4.5				2.8	
Urinary urobilinogen	32× (+)		64× (+)				16× (+)	
GSH in erythrocytes(mg/dl)	59.0		54.5				57.0	
GSH stability test	59.0→28.5 (−51.7%)		54.5→20.5 (−62.5%)				57.0→30.5 (−46.5%)	
6GPD activity in erythrocytes	75		58				72	

clinical experiment (cf. Table IV). Domian* (6-sulfanilamido-2,4-dimethylpyrimidine[3]) and phenacetin[8]) are each reported to be as an inducing agent in drug-induced hemolytic anemia. To Case II of my own patients I gave Domian, 4.0 g per day in three doses for two consecutive days. The results were a decrease of erythroctye counts from 3.05×10^6 to 2.20×10^6 per mm³ and of hemoglobin from 9.7 to 7.5 g/dl, accompanied with distinct signs of aggravated hemolysis such as an elevation of icterus index, of serum bilirubin, especially of indirect fraction, and of urinary excretion of urobilinogen. At the same time, G6PD activity, GSH content and GSH stability in erythrocytes showed a marked decrease. One week later these aggravated signs of hemolysis and metabolic disturbances in erythrocytes returned to the level before administration of Domian, in spite of administration of phenacetin 1.0 g per day in three divided doses for four consecutive days. This indicates that phenacetin in this dosage had no effect on hemolysis and that the spontaneous recovery of aggravated anemia due to Domian administration took place merely by discontinuence of the drug Domian. I tried also the control experiments. The same dosage of Domian

and phenacetin was administered to healthy children, but no significant change of hemolytic signs, GSH content and G6PD activity in erythrocytes occurred. These experimental results seem to suggest that the difference between the congenital nonspherocytic hemolytic anemia and the drug-induced hemolytic anemia is not necessarily essential but rather quantitative, so that the pathogenesis for hemolysis is highly probably the same in both disorders and the difference may be due to severity of gene-penetrance of abnormal trait.

It remains, however, uncertain whether or not so-called "congenital nonspherocytic hemolytic anemia" is an entity from viewpoint of pathogenesis, since a case of "congenital nonspherocytic hemolytic anemia" is at present diagnosed by excluding other well-known kinds of hemolytic anemia such as congenital spherocytosis, erythroblastosis fetalis or the hemoglobinopathies. It remains to be solved whether all cases of congenital nonspherocytic anemia have the above-mentioned metabolic disturbances in erythrocytes. It may be worthy of notice in a veiw of genetics that the two families reported here were of the same area in Yamagata Prefecture.

SUMMARY

Two families of congenital nonspherocytic hemolytic anemia were reported. A metabolic abnormality in the erythrocytes from the patients was demonstrated, consisting of a low content of GSH, an abnormality in the GSH stability test and a low activity of G6PD. These abnormalities were also found in the erythrocytes from the mothers in a milder degree, while there was no abnormalities in the erythrocytes from the fathers. An aggravation of hemolysis and metabolic disturbances mentioned above was observed following administration of Domian (6-sulfanilamido-2,4-dimethylpyrimidine) to the patient.

It was also argued by way of experiment that the difference between the so-called congenital nonspherocytic hemolytic anemia and drug-induced hemolytic anemia is not essential, but only quantitative, so that both disorders belong highly probably to the same entity of enzymopathy from the viewpoint of pathogenesis of hemolysis.

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Hemolysis and Sulfa-drug

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In previous papers,[1-3] the author reported, in two families of congenital nonspherocytic hemolytic anemia, an enzymatic anomaly of erythrocytes, consisting of a low content of reduced glutathione (GSH), an abnormality in the reduced glutathione stability test, and a low activity of glucose-6-phosphate dehydrogenase (G6PD). The author[2] reported that the administration of Domian* (6-sulfanilamido-2,4-dimethylpyrimidine) to the patient with nonspherocytic hemolytic anemia resulted in aggravation of hemolysis accompanied with further decrease of G6PD and GSH in the erythrocytes. From these observations the author designed to investigate the relationship between Domian and the metabolic anomaly of erythrocyte mentioned above. In this respect, there are several possibilities supposed: 1) Domian may inhibit G6PD activity in erythrocytes, 2) it may inhibit glutathione reductase activity in them, 3) it may accelerate oxidation of GSH in them, or 4) it may accelerate oxidation of TPNH* or DPNH** in them. Since both G6PD and glutathione reductase in erythrocyte are a conjugate reaction dependent on TPN⇄TPNH,[1),4)] any one of above possibilities may result in a decrease of the GSH in erythrocytes which substance is probably essential for structual integrity of erythrocyte.[5)] With the above-mentioned suppositions in view, the following 6 experiments were performed.

Experiment I :- The effect of Domian on activity of G6PD and of glutathione reductase was in vitro investigated, using hemolysate from normal human erythrocytes; the hemolysate was prepared as described by Zinkham[6)]. The activity of G6PD was assayed by the method of Zinkham *et al.*,[6)] excepting that incubation was performed at 37°C instead of 25°C. The activity of glutathione reductase was assayed by the method of Schreier *et al.*[7)] In controls an equimolar sodium chloride was used in place of Domian. As is shown in Fig. 1, Domian had a definitely inhibitory effect on G6PD activity at the concentration of 3.75×10^{-3}M, while it had no significant effect on glutathione reductase in the same con-

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TPNH*=reduced triphosphopyridine nucleotide

DPNH**=reduced diphosphopyridine nucleotide

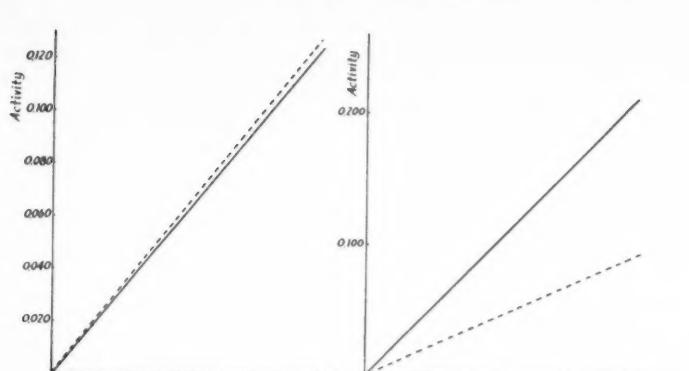


Fig. 1. Effect of Domian on G6PD and glutathione reductase (Experiment I)
..... With addition of 3.75×10^{-3} M Domian
—— Without addition of 3.75×10^{-3} M Domian

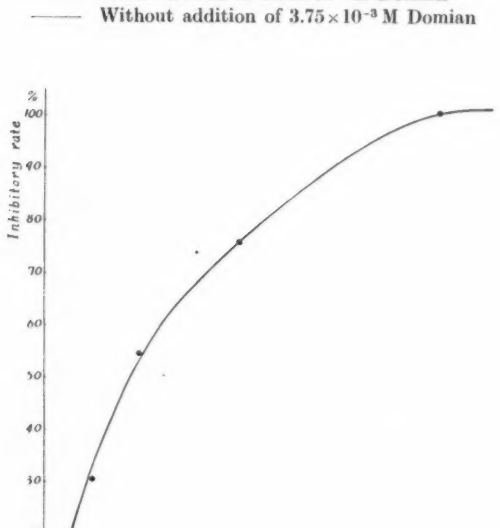
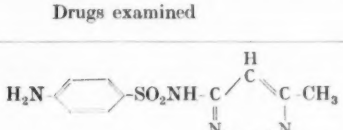
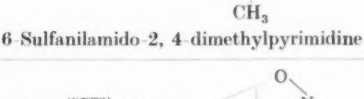
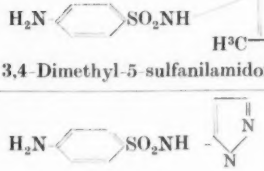
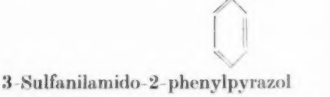
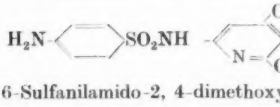
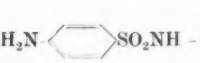
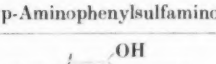


Fig. 2. Inhibitory effect by different concentration of Domian on G6PD (Experiment I)

centration. Fig. 2 shows the inhibitory effect of different concentrations of Domian on G6PD activity. The inhibitory effect on G6PD activity was tried

TABLE I. Inhibitory Effect of Sulfa-drugs on G6PD.

Drugs examined		Inhibitory effect %
Domian	 6-Sulfanilamido-2, 4-dimethylpyrimidine	54
Thiasin	 3,4-Dimethyl-5-sulfanilamidoisoxazole	50
Merian	 3-Sulfanilamido-2-phenylpyrazol	56
Abcid	 6-Sulfanilamido-2, 4-dimethoxypyrimidine	52
Sulzol	 p-Aminophenylsulfaminothiazole	54
Pasnal	 Sodium p-aminosalicylate	30
Sulfanilic acid		23
Sodium benzoate		0
Glutamic acid		0

Concentration of drugs used : 3.75×10^{-3}M

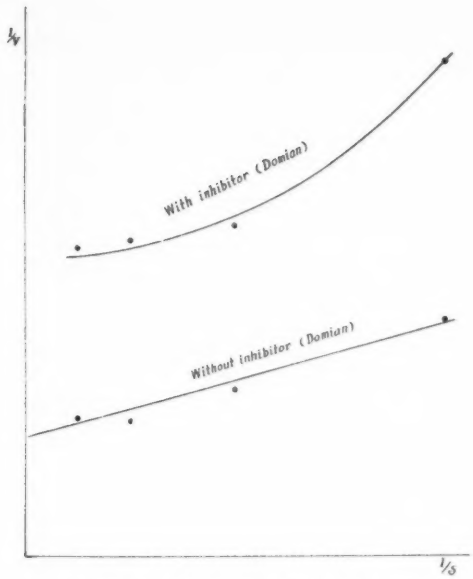


Fig. 3. Lineweaver-Burk's plot

by using various sulfa-drugs other than Domian. It was observed that a series of sulfa-drugs had in vitro an inhibitory effect on G6PD (cf. Table I). Fig. 3 shows a Lineweaver-Burk's plot for inhibition by Domian on G6PD activity. Since there is a distinct difference in maximal velocity of enzyme activity between "with inhibitor" and "without inhibitor", the inhibition is thought to be non-competitative against substrate (glucose-6-phosphate). Fig. 4 shows the inhibition rate by Domian on G6PD activity in different concentrations of enzyme on the one hand and at a fixed concentration (3.75×10^{-3}M) of Domian on the other. A tendency to a decrease of the inhibition rate, in proportion with increase of enzyme concentration, was observed. This may mean that the inhibitory effect by Domian will be manifested under the deficient condition of G6PD.

Experiment II:— 10% suspensions of normal human erythrocytes in isotonic saline-phosphate buffer (pH 7.4) were incubated, with shaking of 70 cycles per minute, at 37°C for 30 minutes with or without addition of 7.50×10^{-3}M Domian. GSH content and G6PD activity in erythrocytes were determined before and after incubation. GSH content in erythrocytes was determined by the method of Gruenert and Phillips.[8] In case of adding Domian, a remarkable decrease of G6PD activity and a milder but significant decrease of GSH

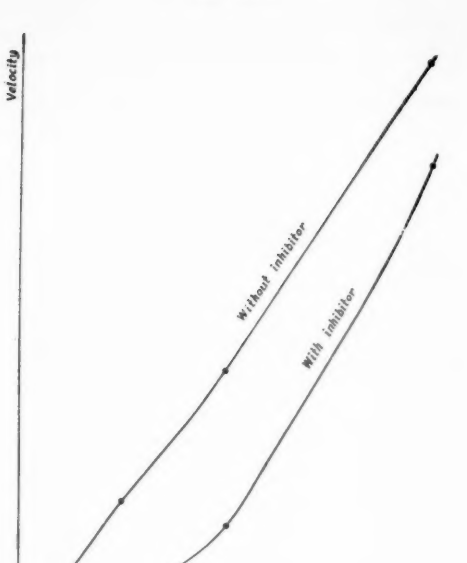


Fig. 4. Inhibitory effect of Domian on G6PD (Experiment I)
Concentration of Domian: 3.75×10^{-3}M

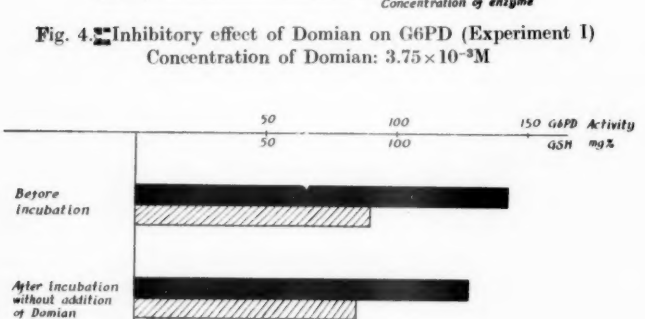


Fig. 5. Inhibitory effect of Domian on G6PD in erythrocyte (Experiment II)

content in erythrocytes were observed (cf. Fig. 5). On the other hand, no significant differences of G6PD activity and of GSH content in erythrocytes were found in case of no Domian addition. These experimental results will give us

the impression that Domian might attack G6PD activity at the first step, resulting in decrease of GSH because of deficient TPNH.

Experiment III : — 10^{-3}M GSH in phosphate buffer (pH 7.4) was incubated, with shaking of 70 cycles per minute, at 37°C with or without addition of Domian (3.75×10^{-3}M or 7.50×10^{-3}M). At intervals of 0, 1/2, 1, 2 and 4 hours during incubation, the concentration of GSH was measured. The results (cf. Fig. 6)

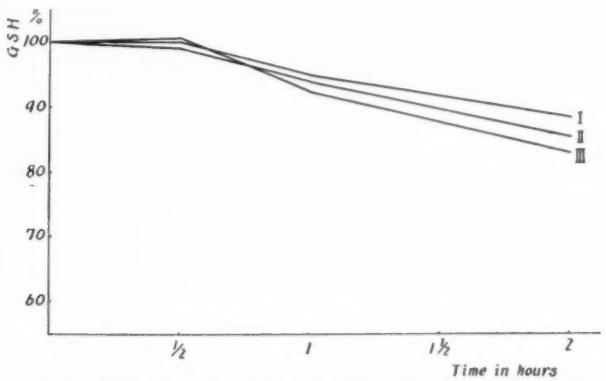


Fig. 6. Effect of Domian on GSH oxidation (Experiment III)
Curves: I; Without addition of Domian. II; with addition of 3.75×10^{-3}M Domian. III; with addition of 7.5×10^{-3}M Domian. Concentration of GSH before incubation was expressed as 100%.

indicated that there was no significant difference of GSH concentration between an addition of Domian and no addition. This will mean that Domian has in vitro no effect on oxidation of GSH.

Experiment IV : — 10^{-3}M TPNH or DPNH in phosphate buffer (pH 7.4) was incubated, with shaking of 70 cycles per minute, at 37°C with or without addition of Domian (3.75×10^{-3}M or 7.50×10^{-3}M). At intervals of 0, 1/2, 1, 2 and 4 hours during incubation, the concentration of TPNH or DPNH was measured spectrophotometrically at 340 mμ. The results (cf. Fig. 7) showed that there was no significant difference of concentration of TPNH or DPNH between an addition of Domian and no addition. This will mean that Domian has in vitro no effect on oxidation of TPNH or DPNH.

Experiment V :— Normal human erythrocytes, which had been washed three times with isotonic saline, were diluted with isotonic saline-phosphate buffer (pH 7.4) to make a 2% suspension. The suspension was incubated, with shaking of 70 cycles per minute, at 37°C for one hour with or without addition of 4×10^{-3}M Salyrgan®. After incubation the suspensions were centrifuged and the hemo-

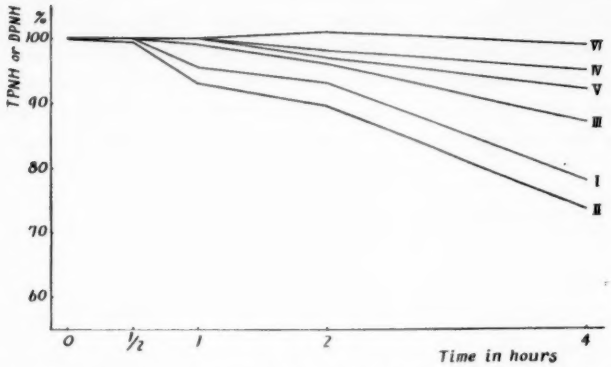


Fig. 7. Effect of Domian on oxidation of TPNH or DPNH (Experiment IV)
Curves: I; THNH only. II: TPNH+3.75×10^{-3}M Domian. III; TPNH+7.5×10^{-3}M Domian. IV; DPNH only. V; DPNH+3.75×10^{-3}M Domian. VI; DPNH+7.5×10^{-3}M Domian. Concentration of TPNH or DPNH before incubation was expressed as 100%.

globin concentration in the supernatant was measured colorimetrically by oxyhemoglobin method. Salyrgan, in the concentrations used, was found to be without significant effect on the absorption spectrum of oxyhemoglobin. In the controls, an equimolar sodium chloride was used in place of Salyrgan. The results obtained are shown in Table II. A remarkable hemolysis during the

Table II. Hemolytic Effect by Salyrgan (Experiment V).

No.	Salyrgan	Glutathione	Hemolysis %
1	(−)	(−)	0
2	4.0×10^{-3}M	(−)	13.5
3	(−)	4.0×10^{-3}M	0
4	4.0×10^{-3}M	4.0×10^{-3}M	0
Complete hemolysate			100

incubation took place by addition of Salyrgan, while no hemolysis occurred in case of no addition of Salyrgan. The hemolysis caused by incubation with Salyrgan was completely protected by the previous addition of an equimolar concentration of GSH.

This hemolytic effect by organic mercury compounds and the protection of the hemolytic effect by GSH were observed by Benesh and Benesh,[9] using sheep erythrocytes. The present author could demonstrate the same phenomenon in human erythrocytes. This experimental results—with Salyrgan that is

known to high affinity for sulfhydryl group in GSH—will indicate that GSH is essential for the maintenance of the intact erythrocyte structure.

Experiment VI :— The suspensions of normal human erythrocytes were prepared in the same way as in *Experiment V*. The incubation, with shaking of 70 cycles per minute at 37°C for two hours, was performed in four different conditions (Nos. 1, 2, 3 and 4) as will be seen from Table III. The grade of

Table III. Hemolytic Effect by Domian (Experiment VI).

No.	Domian	Condition	Hemolysis %
1	(−)	Incubation for two hours	0
2	4×10^{-2}M	Incubation for two hours	0
3	(−)	Addition of 2.2×10^{-3}M Salyrgan after one hour of incubation, and further incubation for one hour	0
4	4×10^{-2}M	Addition of 2.2×10^{-3}M Salyrgan after one hour of incubation, and futher incubation for one hour	6.50
Complete hemolysate			100

hemolysis was measured in the same way as in *Experiment V*. Sample in which an equimolar sodium chloride replaced the test substances served as the control (No .1). A remarkable hemolysis was observed in No. 4, while no hemolysis took place in No. 3. These results will indicate that the erythrocytes previously incubated with Domian, which would, as has been shown in Experiment II (cf. Table II), bring about a decrease of G6PD activity and of GSH content, became so fragile as to lead to hemolysis. Although the cases of hemolytic anemia following the administration of sulfa-drugs have been reported[10-12], it is not yet clear why a sulfa-drug should bring about hemolysis. Through the experimental results described in this paper, it was elucidated that sulfa-drugs have in vitro an inhibitory effect on G6PD activity in erythrocytes, thus resulting in decrease of GSH in erythrocytes. Since the maintenance of GSH is dependent on G6PD activity in erythrocytes,[1),13)] it may follow that the decrease of G6PD activity will result in decrease of GSH in erythrocytes. In normal individuals, G6PD activity in erythrocytes may not be in vivo affected by an usual dosage of sulfa-drug, because enzymes such as G6PD are thought to exist in an ample amount in normal erythrocytes. However, when such individuals as deficient in G6PD in erythrocytes are exposed to sulfa-drugs, the inhibitory effect by the drugs on G6PD activity may be manifested, thus resulting in a further decrease of GSH and in aggravation of hemolysis.

SUMMARY

Basing upon the observations of hemolytic effect by the administration of Domian to the patients with erythrocytes deficient in G6PD and in GSH, a relationship between sulfa-drug and metabolism in erythrocytes was investigated. The experimental results obtained are as follows.

1) Domian has in vitro an inhibitory effect on G6PD activity but no effect on glutathione reductase activity.

2) A series of sulfa-drugs have in vitro a similar effect on G6PD activity as that by Domian.

3) Inhibition of G6PD activity by Domian may be noncompetitative against glucose-6-phosphate.

4) Inhibition rate of G6PD activity by Domian tends to decrease in proportion with increase of concentration of the enzyme.

5) Domian has in vitro no effect on oxidation of GSH.

6) Domian has in vitro no effect on oxidation of TPNH or DPNH.

7) The hemolysis of human erythrocyte caused by incubation with Salyrgan is completely protected by addition of an equimolar solution of GSH.

CONCLUSIONS

When such individuals as deficient in G6PD in erythrocytes are exposed to sulfa-drug, an inhibitory effect by the drug on G6PD in erythrocytes may be manifested by such a dosage of the drug that normal individuals are not affected. Then a block of G6PD activity in erythrocyte caused by the drug in those patients may result in a further decrease of GSH in erythrocytes due to deficiency of TPNH and in aggravation of hemolysis.

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Histopathological Studies on Autopsied Cases of Stomach Cancer Treated with Anticancerous Drugs

By

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Researches on anticancerous drugs are advancing by leaps and bounds, innumerable drugs having been subjected to clinical trial already,[1)2)3)4)5)6)7)8)9)10)11)12)] but no ideal anticancerous drug has been yet announced. For discovery of a better anticancerous drug, it seems indispensable to rescrutinize the effect of such agents in current use on the malignant neoplasms in human body more fundamentally, to grasp the demerits as well as the merits of the individual preparations advertized as anticancerous. From such a veiw point, the present author made a histopathological investigation of the effect of administering of such drugs, using specimens obtained from autopsied cases of stomach cancer, the cancer most frequent among the Japanese, as materials. In the following, a summary report on the outstanding results is presented.

MATERIALS AND METHODS

The materials of this study were sampled from corpses autopsied within 12 hrs. after death, to minimize the effect of postmortem alterations, among the 123 cases of stomach cancer autopsied in 1954–60, at the Pathological Laboratory of this university; the specimens from 20 of stomach cancer cases that had received anticancerous drugs for more than a week and died within one month after discontinuance of the administration were examined as the treated cases, and 27 specimens from such cases that had no administration of anticancerous drug were taken up as controls. As shown in Tab. I, the treated cases are divided into groups by the varieties of the administered drugs: 4 cases of Nitromin group, 1 case of RC_4 group, 2 cases of Azan group, 3 cases of Mitomycin group, 8 cases of Carzinophilin group, 1 case of Predonin group and 1 case of X-ray deep-irradia-

橘　芳郎

Table I

	Case	Sex	Age	Medication	Dosis/day and method of administration	Total dosis	Survival period after withdrowal medication (day)	Operation	The other
Alkylating substances	1	M	55	Nitromin Isotop gold	20–50mg i.v. 30mC i.p.	1000mg 2×	14	no	
	2	M	58	Nitromin Isotop gold	25–50mg i. v. 30mC i. p.	825mg 1×	10	no	
	3	M	57	Alanine-Nitrogen-Mustard Etoxyl-Nitromin	5–10mg i. v. 50mg	120mg 150mg	7	no	
	4	F	25	Alanine-Nitrogen-Mustard	5–10mg i. v.	90mg	0	no	
	5	F	70	RC_4	50mg i. v.	500mg	30	no	
Antibiotics	6	F	48	Alanine-Nitrogen-Mustard Cyclohexyl-Nitromin Carzinophilin	5–10mg i. v. & i. p. 20mg i. v. 5000Unit. i.v. & i.p.	1655mg 120mg 330000U.	4	no	ACTH 870mg
	7	M	47	Carzinophilin	5000Unit. i.v. & i.p.	215000U.	15	op	ACTH 12.5mg
	8	F	47	Carzinophilin	5000Un it.i.v. & i.p.	200000U.	30	op	
	9	M	65	Carzinophilin	50000 Unit. i.v.	100000U.	0	no	
	10	F	60	Carzinophilin	5000 Unit i.v.	100000U.	5	op	
	11	M	48	Carzinophilin	5000 Unit. i.v.	98000U.	24	op	ACTH 220 I.U.
	12	F	68	Carzinophilin	5000 Unit. i.v.	85000U.	0	op	
	13	M	54	Carzinophilin	5000 Unit. i.v.	50000U.	20	no	
	14	M	44	Mitomycin Isotop gold	2mg i.v. 30mC i.p.	84mg 3×	20	op	
	15	M	64	Mitomycin	2mg i.v.	68mg	1	no	
	16	M	55	Mitomycin	2mg i.v.	42mg	30	no	
Anti metabolite	17	F	48	Azan	40-80mg i.m.	2000mg	1	no	
	18	M	62	Azan	80–160mg i.m.	1440mg	12	op	
The others	19	M	60	Röntogen		6500r	30	no	
	20	M	48	Predonine	10mg Per os	300mg	7	no	

* i.v.: intravenous
i.p.: intraperitoneal
i.m.: intramural

tion group (control).

Of each specimen, the central and the peripheral part of the tumor were cut out, and besides, sections were sampled from the liver, the kidney, the spleen and the adrenal glands, regardless of presence or absence of metastasis, to prepare paraffin embedded sections. Staining was done with hematoxylin-eosin in most cases, but mucicarmine, PAS, Azan-Mallory's and Masson's trichrome stainings were also applied when necessity arose.

RESULTS

The materials in this study were all sampled from autopsied cases, as stated above. Since the administration of anticancerous drugs was began in the terminal stadium of stomach cancer in all the cases, and the administration had to be suspended on account of leucopenia and some such side-effects, the therapy was mostly discontinued within a month in most cases, the case with the therapy continued for 4 months being quite exceptional. In some cases, the anticancerous drug treatment proved temporarily effective, but the tumor was found reaggravated at death. Thus, the exact morphological effects of the drugs were not observable in all the cases, and minute caution was required in rightly evaluating the efficacy, but in studying the control specimens in collation, some findings suggestive of the efficacy of the anticancerous drugs could be morphologically noted. The results obtained are so variegated that generalization seems difficult enough, but the morphological characteristics suggestive effect of the efficacy of anticancerous drugs may be summarized as follows:

1) Atrophy, degeneration and necrosis of cancer cells: As is well known, such necrosis and disintegration of cells in the central part of cancerous tissues are often observable also in cases of cancer not treated with anticancerous drugs and always seen in particularly large cancerous focus and solid carcinoma, but in the specimens from the cases that received such drugs, these changes in cells were particularly notable. But after all, such differences are only quantitative, and not at all essential. These changes are probably histological occurrences due to circulatory disturbance. But in the peripheral zones of the cancerous tissues, namely, the outermost part of proliferating cancer cells in contact with the healthy surrounding tissue and the part in direct contiguity with the stroma, some changes apparently due to the direct effect of anticancerous drugs could be observed[13]. Some of the cancer cells in these sites were found often atrophied, and also loosened in epithelial arrangement, sometimes necrotized and disintegrated. This finding was hardly observable in the control specimens and may be regarded as specifically due to the effect of anticancerous drugs. These changes were more apparent in the parts exposed to closer contact with the drugs carried by blood stream. Accordingly, such changes were usually more marked in the cases of scirrhous cancer, rich in granulation tissue, where the opportunity for

the cancer cells to come in contact of the drugs was more frequent, than in carcinoma simplex with scanty stroma. These changes are also more notable in the parts showing marked reaction of young granulation tissue and rich in blood vessels, than in the parts where the cancer cells were infiltrated into the dense connective tissue with poor capillaries; in the gastric wall, the most prominent changes were observed in the cancer infiltration foci of the submucosal and subserous tissues rich in blood capillaries and apt to show inflammatory reaction.

The differences of findings by the varieties of the administered drugs depend upon the differences of dosage and duration of the treatment but we may say that the Nitromine derivatives are strong in their effect of causing dissolution and degeneration of peripheral cancer cells and this effect may be deemed as characteristic to these drugs, together with the changes they cause in the stroma, as described below. In the cases treated with antibiotics, the accelerated necrosis of cancer cells, as observed also in the control cases, was notable. So, though differences in the degree of changes of cancerous foci following administration of different drugs may be inevitable, in the cases wherever the anticancerous effect was clinically discernible, changes of both the types above were usually equally notable, the cancer cells infiltrating the subserous tissue (and sometimes the submucosal tissue) being particularly subject to the effect of drugs, tending to lose their epithelial arrangement and to show the form of dissociated and scattered histiocyte-like cells.

2) Difference in effect of anticancerous drugs on different histological types of cancer: The above mentioned changes were observed in cancer cells of different types after drug-treatment, at least in restricted areas. On the other hand, the effects of drugs not sufficiently strong to cause disintegration or necrosis of cancerous tissues, that is, the milder effects, took more or less different forms according to the histological types of the cancer specimens.[14)15)16)] In adenocarcinoma cases, the disparity in size and form of the affected cancer cells were notable, and the frequency of appearance of cell forms as if suspended in the course of atypical mitosis and oversized nuclei seemed to multiply. On the other hand the degenerative changes such as vacuolation become more frequent and the mucus production usually tended to rise, so that in the cells of gelatinous cancer and the cancer type showing diffusive infiltration of signet cells inherently showing large mucus production, the tendency to gelatinous degeneration was amplified. Adenocarcinoma and scirrhous cancer partly mixed with gelatinous cancer are conspicuous. In the so-called carcinoma simplex or medullary carcinoma, the inequality of size and form of the nuclei and the cytoplasms show the tendency of intensifying, and in particular, in the case where medium- or large-sized cancer cells are in dence arrangement, the cells in the central part are enlarged and often take the form of squamous epithelia. It has been

stated above that scirrhous carcinoma is receptive to the influence of anticancerous drugs; scirrhous cancer cells under the influence of such drugs show besides the otherwise described changes also increase in size including giant cell formation. The cell degeneration and atrophy here resemble the changes appearing in the sites with dense stroma in the control specimens, but in the specimens of the treated cases, in spite of the looseness of the stroma or the intense activity of granulation tissue, the cells are often in the course of dissolution, atrophy and destruction, and cells in atypical mitosis and giant cells are conspicuously perceptible.

The above mentioned changes of cancer cells are not specific to the specimens of drug-treated cases and are more or less observable in the control specimens as well, but as these changes appear more frequently and in more multiple forms in the former specimens, we may definitely attribute them to the effect of anticancerous drugs.

3) The effect of anticancerous drugs on the tumorous stroma: The cancer may be classified by the criterion whether the histological structure consists mainly of parenchyma or of stroma into carcinoma medullare, simplex and scirrhosum. In any case, the stroma more or less distinctly shows the features of granulation tissue and reveals more or less intense inflammatory reaction.[17][18] Such findings are especially notable in stomach cancer. Now, administration of anticancerous drugs affected the picture of carcinomatous stroma in the following manner. After Predonine administration the inflammatory cells were reduced in number, the stroma was turned into an edematous tissue and was furthermore partly hyalinized. In the granulation tissue, the stroma after alkylating substances and Azan administration were edemotaus and loss of cell reaction was notable, so that inflammatory cell reaction was weakened even near the tip of tumorous proliferation and only an edemotaus connective tissue surrounds the tip. After rather long term administration of these drugs, such changes are mixed with some hyalinization findings. After antibiotics administration, the inflammatory cells are comparatively well preserved in the stroma, fibrosis becomes perceptible and when obsolete hyalinization sets in. The highest proliferation of the connective tissue was found in the cases subjected to X-ray irradiation. Isotopic gold is usually applied intraperitoneally in combination with systemic dosage of the other drugs, and its effect was apparent only inside the peritoneum, probably acting in the characteristic manners of neoformation of blood capillaries and proliferation of fibroblasts, besides causing atrophy, degeneration and disappearance of cancer cells.[19]

4) Influence of anticancerous drugs on metastasized foci: The author's specimens were classified into the types of peritoneal, hepatic and lymphnodal, according to their mode of metastasis, as proposed by Enjoji[20] and the effect of the anticancerous drugs on the respective metastasized foci was examined. As

shown in Tab. II, in comparison with the untreated control cases, the rate of metastasis to the liver was lower in the treated peritoneal type cases, and cancerous peritonitis and metastasis to the adrenal glands were more frequent in the treated lymphnodal type cases. These findings were in good agreement with the results of histological examination.

Table II.

	Type of metastasis		Total	Infiltration	Peritoneal dissemination	Hematogenous metastasis				Lymphogenous metastasis	
						Liver	Pancreas	Lung	Adrenal gland	Regionar	The other
Non-operating group	Peritoneal type	Treated cases	5	4	5	1	3	0	4	4	1
		Control	7	6	7	5	2	0	1	5	2
	Hepatic type	Treated cases	1	0	0	1	0	1	0	1	1
		Control	6	1	2	6	1	4	0	6	2
	Lymphnodal type	Treated cases	7	2	4	4	1	1	5	7	3
		Control	8	2	1	4	3	2	2	8	5
	No metastasis	Treated cases	1	0	0	0	0	0	0	0	0
		Control	0	0	0	0	0	0	0	0	0
Operating group	Peritoneal type	Treated cases	2	1	2	1	0	0	0	2	0
		Control	2	0	2	2	0	0	0	0	0
	Hepatic type	Treated cases	3	1	1	3	2	0	2	3	2
		Control	0	0	0	0	0	0	0	0	0
	Lymphnodal type	Treated cases	1	1	1	0	0	0	1	1	0
		Control	4	1	1	3	1	1	2	4	2

As detailed below administration of anticancerous drugs is often followed by formation of small necrobiotic or necrotic foci, reflecting the effectiveness of the agents against the foci in the liver as in the other organs. The tumor cells were particularly often found in atrophy, degeneration or necrobiosis near the periphery of the cancer where it touches the liver parenchyma, sometimes the hepatic cell cord being found in direct contact with the necrobiotic cancer cells in the metastatic focus. These findings are in good agreement with what may be expected from the characterjstic courses of the portal vessels. It may be easy to understand that such changes appearing only as phenomena in the terminal stadium such as metastases to the liver should show low frequency in the treated cases. The anticancerous drugs tested seemed to have no marked effect on the metastatic foci in the lymph nodes and the lymphatic vessels.

Upon close examination, cancerous peritonitis is found caused by cancer cells from the stomach either reaching the focus in the serous membrane in a row through the tissue interstices and disseminating in the peritoneal cavity, or reaching the subserosal tissue in the form of emboli in the lymphatic vessels and thence spreading to the serosa. In these cases, even when the cancer cells

infiltrating the subserous tissue are under strong effect of the agents, the cancerous emboli in the lymphatic vessels are not much affected.

Among the 8 lymphnodal type cases treated with anticancerous drugs, metastases to the adrenal glands were observed in 6 cases. Of these, in 2 cases the metastasis came directly from the above mentioned cancerous peritonitis, and in the 4 others it was of hematogeneous nature. But metastasis to the adrenal gland was found in 2 of the 4 treated hepatic type cases, against none in the 6 non-treated cases of type, and in 4 of the 7 treated cases against 1 in 9 non-treated cases of peritoneal type, in both cases the frequency being higher in the treated cases. Such metastases have been perhaps facilitated by the congestion in the adrenal glands and excitation and exhaustion of their often observable after anticancerous drugs administration, as mentioned below. It is of some interest that while the frequency of hematogeneous metastasis is nearly uniform in all other organs, it is exceptionally high in the adrenal glands. The nearly entire absence of signs of the effect of anticancerous drugs in the histological picture of the metastatic cancer in the adrenal gland also suggests that the finding is not unconnected with the administration of the drugs.

The representative hematogeneous metastasis is that to the lung, and the anticancerous drug therapy seems to have no influence on the frequency of such metastasis; the effect appearing in the metastatic foci in the lung as examined histologically is seemingly weaker than that in the liver. This is perhaps not only due to the fact that such a metastasis is a terminal phenomenon, but also due to the low frequency of opportunities for the cancer cells to come in contact with the anticancerous drugs owing to the forms of emboli in lymphatic vessel or cancerous alveolitis characteristic to pulmonary metastases. In only 5 of the said 20 cases, bon emarrow examination was performed; in them, nodullar and hemorrhagic necroses were observable as signs of the effect of anticancerous drugs, not only in the parts affected by metastases, but also in any part of the bones including the marrow and the cortex, forming small nodules. Such changes, however, were sometimes present also in the non-treated controls, so that they may be attributable to the difference of distribution pattern of the arteries of the bone, but it was undeniable that the frequency and the scope of such changes were larger in the treated cases. Besides, signs of the effect of anticancerous drugs similar to those on the stomach wall were also observed in the cancer cells in the bone marrows.

5) Different effects on the cancer cells after different administrations: The anticancerous drugs are most frequently injected intravenously, but Azan is administered by intramuscular injection, and in some cases, intraperitoneal application is also used in combination. In my cases, 3 had been intraperitoneally injected with isotopic gold, 1 with Carzinophilin and 1 with Carzinophilin plus Alanin-nitrogen mustrard. After such intraperitoneal administration,

decrease of ascites was clinically confirmed, but histologically a rather strong effect to cancerous peritonitis was noted. The case with injected isotopic gold has been discussed in the above. In the Carzinophilin-treated cases, not only the tendency to degeneration and atrophy of cancer cells in the serous membrane was enhanced, but besides a finding of nonspecific peritonitis slight inflammatory cell infiltration and poor blood capillary formation was observed, and sometimes fibrous incrassation of the serosa was often present. The effects of the agent on the metastasized cancer in the liver was stronger following simultaneous intraperitoneal administration.

6) Changes in the other organs and tissues following administration of anticancerous drugs: Of the clinical side-effects of anticancerous drugs, the changes in the blood picture are most conspicuous (Wada *et al.*[21]). In my cases, the bone marrow examinations were made only in several cases of this study and therefore the systematic investigations of the effect of the agents on the hematopoietic organs were unfortunately not performed. In the lymphatic system, the lymph organs including cortical nodules of the lymph nodes and the spleen were found generally atrophied but not too highly impaired. The peripheral vascular system was rather severely affected, in particular, the side-effect of Azan on the system being already often noted (Yamamoto *et al.*,[4] Morita *et al.*[22] and Iwai[23]), and in the one Azan-treated case of mine, too, hemorrhagic necrosis was very obvious in the lungs, liver and kidneys, etc. In the cases treated with Nitromine derivatives, typical emboli were observed sometimes in the spleen and kindey, etc., besides decrease of free cells in the spleen, edema of the renal stroma, dilatation of the tubuli, edema, swelling and cylinder formation in the mesangium of the glomeruli in the kidney in some cases. In the exceptional case (No. 6), who had suffered from severe thrombophlebitis from the vena brachialis to the vena jugularis and died from advanced hemorrhagic infarct in the bilateral lower lobes of the lung due to emboli in the pulmonay artery, treatment with 1,655 mg of Alanin-nitrogen mustard and 330,000 units of Carzinophilin had been applied. Though we must be cautious in attributing all such vasucular lesions to the agency of anticancerous drugs, such findings must be given due attention. In the liver of this case, besides edema, swelling of the liver cells and such diffusive changes, necrobiotic or necrotic foci were found in small nodular form, as mentioned already.

There have been some reports on the adrenal cortex in patients with cancer (Hamaguchi,[24] Hahn,[25] Setsuda[26]). Generally speaking, in stomach cancer cases, the adrenal cortex is atrophied, particularly thinned down in the fascicular zone but remains somewhat thicker in the reticular zone. In the cases with large retention of ascites, however, the adult glomerulosa was found thickned, but even in such cases, the thickness of the cortex as whole in the control cases was not found increased, while in the cases treated with Nitromin derivatives, the cortical

cells were often subject to swelling, cytolysis and such acute excitation lesions. After antibiotics administration, such lesions were milder and the cytoplasm of the cortical cells was densely granulated, and the lipoid was obviously diminished in all the cases.

Reports on the lesions in the solid organs have been published (Iijima *et al.*,[29] Kubota[30]). In the author's cases too, such lesions were always observed, but apparently more notable in the cases treated with alkylating substances than in those with antibiotics. But the severity of such lesions is more or less different according to the mode of drug administration, and it is particularly notable that in the cases pretreated with ACTH, exhaustion symptoms in the adrenal cortex are kept low and its change was limited to thickening. This finding is very interesting when it is considered in relation with problem of using anticancerous drugs and adrenocortical preparations in combination.[21]

The effect of anticancerous drugs was also found in secondary inflammation foci, and also in the histological nature of terminal bronchopneumonia. For example, after the administration of alkylating substances, mobilization of polynuclear leucocytes and formation of granulation tissue in the inflammatory foci were limited and exudation of such foci was often observed, but after antibiotics administration the original antibacterial effect seemed apparent[32].

DISCUSSION

Evaluation of the effect of anticancerous drugs on cancer, using autopsy specimens, [21][31][34][35][36][37][38][39][40] is a very difficult task, since the cells of malignant tumors are naturally very apt to degeneration and disintegration and strict precaution is called for in such a study. In the present study, the cases were not seldom where the clinical effect of decrease of ascites and improvement of subjective symptoms had been apparent, but the picture of such improvements were hidden at the autopsy beneath the pictures of exacerbated tumor that had caused the death of the patient or under the aftermaths of side-effects of the agent. In the 20 cases cited in this study, clinical efficacy had been noted in 6 cases. In these cases, where should we seek the morphological substrata of the apparent improvement of clinical symptoms before death (the cause of which we will not touch upon here) ? Upon studying specimens from autopsy cases after Sarcomycin treatment, Hirafuku[1] stated that the morphological foundation of the clinical improvement, especially, contraction and extinction of the cancer can be sought in nothing but the nonspecific changes consisting in necrosis, degeneration and hemorrhage in the tumorous tissue, and finding in the surrounding tissue. He also asserted that only when the action mechanism of the anticancerous drugs has been elucidated, by help of biopsy of spontaneously cured cases of malignant neoplasms, such morphological changes can be rightly interpreted. Among the 27 control cases in the present study, no one was cured

spontaneously, but in all these cases, upon closer examination, various degeneration processes including necrosis in the cancer tissue were always observed as well as the process of active proliferation. But such degenerative processes were limited in scope, and in the stomach wall as well as the metastasized foci, at the tip part of proliferation between the tumor and the surrounding tissue, active cancer cells were always observed.

The stroma of stomach cancer is found in different conditions from case to case, but always showed the features of granulation tissue more or less notably, and usually accompanied inflammatory cell reaction. The cells of cancerous parenchyma showed findings of many types by cases, but in one individual case, the proliferating cancer cells are usually found in rather uniform shape and size. Sometimes, giant cells are sporadically in existence, but in no case could be seen the picture of large-cellular or polymorphcellular carcinomas as often observed in carcinomas of the thyroid and pancreas.

The above-mentioned changes in the cancer cells were confirmed as the results of examination of specimens of the treated cases, always with such histological features and other findings observed in non-treated controls in mind. As retrogressive changes, degeneration and necrosis of cells were observed not only in the central part of the cancer foci but also in the marginal parts adjacent to the stroma and the surrounding tissue. In the cells of stomach cancer themselves, polymorphcellular formation, gelatinous degeneration and squamous cell metaplasia were notable, inflammatory cell reaction in the stroma was deficient and histological changes from formation of edematous connective tissue to hylaline fibers were observed; the frequency and the extensiveness of such changes may be taken to betray the effect of anticancerous drugs.

In animal experiments on the effect of anticancerous drugs, the problems of the difference of effect by variety of the drugs and the site subject to its action have been chiefly taken up (Ishidate[1], Umezawa[1], Hata *et al.*[1]). But with such postmortem specimens as examined in the present study, the evaluation of any difference in efficacy must be done with utmost caution and a discussion on the difference of efficacy by the variety of drugs on the basis of histological findings is made even more difficult. It could be ascertained, however, that after administration of Nitromine derivatives, the tendency for the cancerous tissue to lose the epithelial character and to fall into dissolution and degeneration was more in evidence, while after antibiotics administration advance of degeneration and necrosis in spontaneous manner was more marked. In the stroma, inflammatory cell reaction was maintained intact after antibiotics administration, but after Nitromine derivatives administration this reaction was inhibited and the tendency to edematous degeneration was intensified.

Such a difference in efficacy is more apparent upon intraperitoneal administration of anticancerous drugs in the cancerous peritonitis cases, for here the serous

membrane is thickened after antibiotics administration, while it is hyalinized and thinned down after Nitromine derivatives administration, and after administration of isotopic gold the capillary endothelium and the fibroblasts are proliferated and swollen. Upon systemic administration, the effect of anticancerous drugs comes out differently by sites according to the quantity of the drug reaching the site in blood flow. For example, in the stomach wall, the cancer cells infiltrating the submucosal and the subserous tissues rich in blood vessels and granulation tissue are readily exposed to the effect of anticancerous drugs, and in the very cancer tissues, scirrhous cancer rich in stroma is more apt to show the effect of drugs than medullary carcinoma, while in carcinoma simplex the peripheral parts are particularly affected. Next, in the cases of gastric carcinoma the effect of anticancerous drugs was far more notable in the case of metastasis to the liver than that to the lymphnodes, but the infiltration foci around the latter was found subject to the effect. Degeneration and necrosis of cancer cells were often found in metastatic foci in the bone-marrows. These findings suggest that more lymph-affinitive drugs should be selected or the mode of administration should be reconisdered, to assure efficacy of anticancerous drugs against the lymphnode metastases of cancer.

As described above, the author's examinations have led to findings substantiating the effects of reduction of tumor in size and decrease of ascites clinically observed in general as effects of anticancerous drugs, though it cannot de benied that some inequality in the efficacy in causing changes in the cancerous tissues was manifest according to the difference in cancer types and in the variety of drugs applied. On the other hand, in the author's specimens of cancer cases mostly treated with drugs in the terminal stadium of disease where operation was too late or postoperative recurrence was apparent, no effect of the prolongation of life was noted. The side-effects of the drugs cannot be neglected in a discussion on this point.

The liver, as stated above, is comparatively affected by the effect of anticancerous drugs, owing to its free blood flow, but this condition also makes it liable to lesions in the parenchyma due to the drugs. In my specimens, small necrotic foci were often found in the liver.

Leucopenia is often clinically recognized as a disturbance of the hematopoietic organs but sometimes the lymph apparatus in the spleen is atrophied and the free cells in its medulla are decreased. Next, we must specially mention the excitation lesions of the adrenal cortex ; on the one hand, metastases to the adrenal glands become more frequent in the terminal stadium, while on the other, the exhaustion of the adrenocortex results in earlier death.

These side-effect lesions are apparently worse after Nitromin derivatives administration than after antibiotics. It seems, however, that such side-effects may be at least partly excluded by pretreatment with ACTH and such adreno-

cortical preparations. In fact, in the cases of my specimens pretreated with ACTH, nearly no exhaustion of the adrenocortex could be confirmed.

As the patients of stomach cancer treated with anticancerous drugs had all died during the therapy or within a rather short period after the therapy, these may be called cases of no effect of such drugs. But when we recall that in every case of them, some histopathological signs, more or less manifest, of the effect were observed, we may be justified in expecting successful progress in the future of the chemotherapy for carcinoma just recently introduced in clinical practice.

CONCLUSION

20 specimens sampled at autopsy of stomach cancer cases treated with anticancerous drugs were histopathologically studied, with similar specimens from 27 cases not treated with such drugs as controls. The results obtained were in summary as follows:

1. In the drug-treated cases, atrophy, dissolution, necrosis and such non-specific retrogressive degeneration of cancer cells were quantitatively more advanced than in the control specimens. Such changes were particularly observable in the tip of cancerous proliferation, and more notable in scirrhous cancer than in medullary carcinoma or carcinoma simplex.

2. As more mild changes attributable to the effect of anticancerous drugs, disparity in size and form, atypical mitosis, mucous degeneration, giant cell formation and squamous cell metaplasia were more frequently found, but these cahnges were not essentially different from similar changes found also in the controls.

3. In the tumor stroma of the drug-treated cases, generally speaking, the inflammatory cell reaction was reduced and edematous swelling was advanced, in somewhat different manner and degree according to the variety of the anticancerous drugs, as follows:

a) After administration of alkylating substances and Azan, the stroma was highly edematous, the cell reaction was mild, and the degeneration and dissolution of contiguous cancerous tissue was notable.

b) After antibiotics administration the stromatic reaction remained rather well preserved, but coagulative necrosis and variegation of the cancer tissue in original form were advanced.

c) After intraperitoneal application of isotopic gold, characteristic findings were observed in the granulation foci of non-specific peritonitis.

4. All the cases examined were classified into the types of peritoneal, hepatic and lymphnodal, according to their mode of metastasis, and the effect of anticancerous drugs on the metastasized cancer was found generally similar to that on the primary focus, but seemingly subject to the influence of the conditions of blood flow and the mode of drug administration.

5. In the drug-treated cases, the liver showed the changes of edema, cell swelling and small necrotic foci, and the adrenal glands revealed swelling of the cortex and in most cases after Nitromin derivatives administration the acute excitation lesions of swelling and dissolution of adrenocortical cells; after administration of antibiotics such lesions were milder and the cytoplasm was found amplified. In the cases pretreated with ACTH, such lesions were always kept mild, swelling of the cortical cells alone being marked.

6. Findings of embolic necrosis and thrombi probably secondary to vascular lesions were notable in some cases after Azan and alkylating agents.

7. As discussed above, the anticancerous drugs studied in the present work had some effect, sometimes powerful enough, against cancer tissues, but we may expect for further study on the questions of improving the permeability, the mode of administration, preventing the side-effects of the drugs and prolonging the life of the treated patients.

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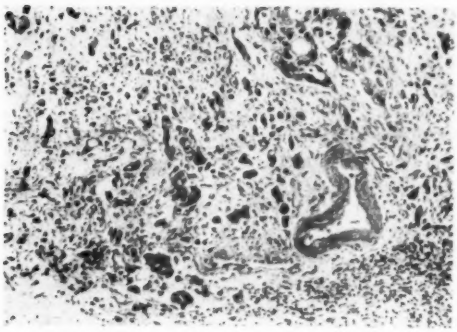

Fig. 1. Specimen after 10 day's treatment with 5–10 mg of Alanin-nitrogen-mustrad per diem (Case No. 4). Lesions in the cancerous tissue infiltrating into the gastric subserosal layer: Essentially this case is adenocarcinoma, but the cancer cells loose often pattern of the epithelial arrangement and show atrophic degeneration as well as atypical giant cell formation.

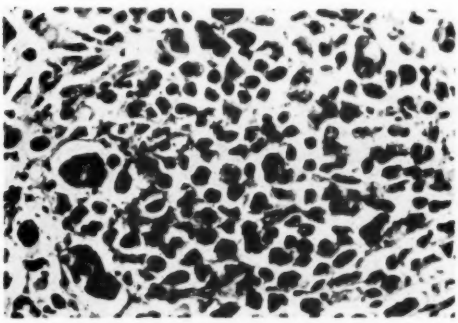

Fig. 2. Specimen after 100,000 units of Carzinophilin (Case No. 9). Essentially this is cubic-epithelial adenocarcinoma but the cancer cells infiltrating into the sumbucosa show dissociation and degeneration and are in a polymorphocellular pattern.

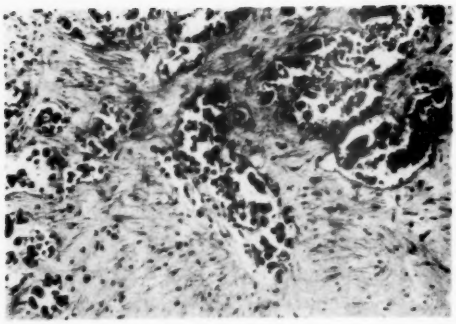

Fig. 3. Specimen after intraperitoneal injection of 1,000 mg of Nitromin and 30 mc of Au^{198} (Case No. 1). Essentially this case adenocarcinoma, but the cancer cells notably changed by variation in size, dissociation, degeneration and necrosis. The stromatic connective tissue is edematous and poor in inflammatory cell reaction.

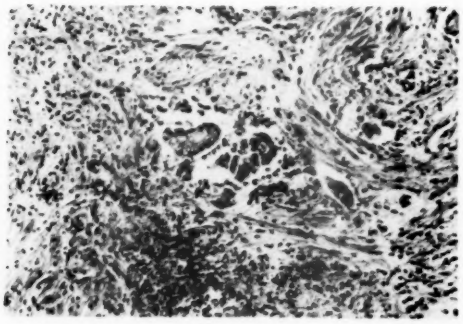

Fig. 4. The same specimen as in Fig. 2. The adenocarcinoma tissue is markedly degenerated and dissolved. Round-cell infiltration obvious in the stroma.

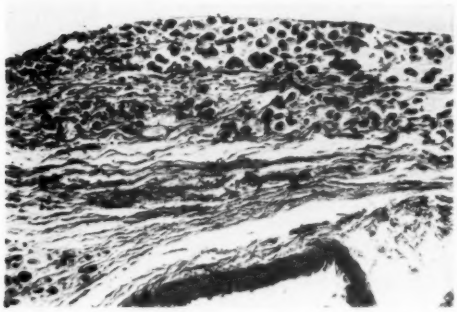

Fig. 5. Specimen after intraperitoneal injection of 1,655 mg of Alanin-nitrogen-mustard and 330,000 units of Carizonophilin (Case No. 6).

The case treated for the longest duration; the cells in the cancerous focus infiltrating into the stomach serosa have become free cells, and show disparity in size, eccentric localization of nuclei and such variegated changes.

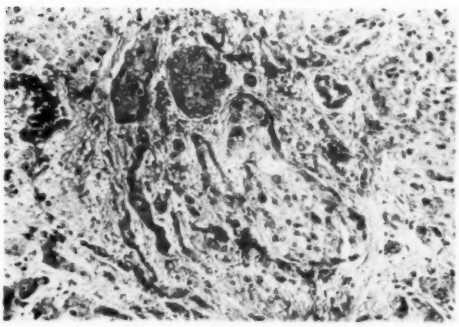

Fig. 6. Specimen after 100,000 units of Carzinophilin (Case No. 10).

Stomach cancer metastasized in the liver. Findings of adenocarcinoma are apparent in the stomach wall, but in the liver, the tendency to squamous cell metaplasia is manifest here.

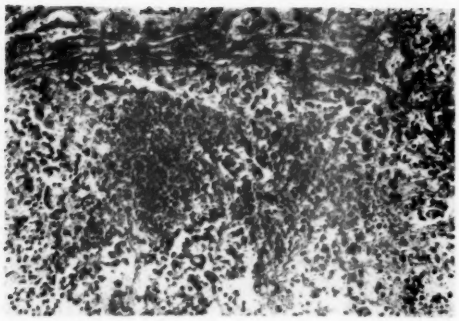

Fig. 7. The same specimen as in Fig. 3.
Stomach cancer metastasized in the liver. Disintegration and necrosis are conspicuous in the cancer focus, particularly so in the marginal part adjoining the hepatic tissue.

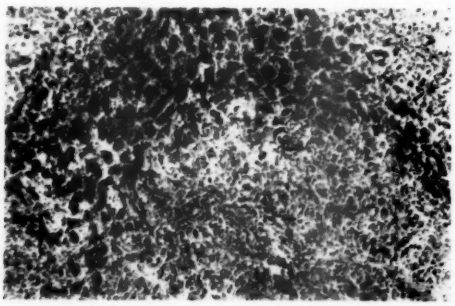

Fig. 8. Specimen after 165,000 units of Carzinophilin (Case No. 7).
Necrosis is conspicuous in the cancer tissue of metastatic focus in the liver adjoining the liver tissue.

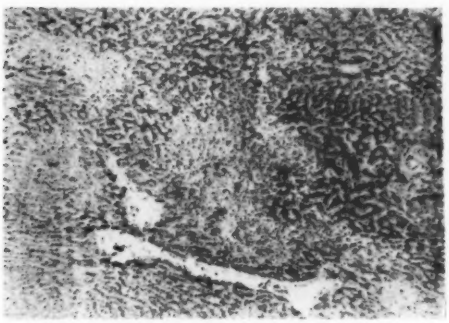

Fig. 9. Liver of the specimen shown in Fig. 1.
Necrotic focus formed at the center of a liver lobule ; edema around the capillaries is also marked.

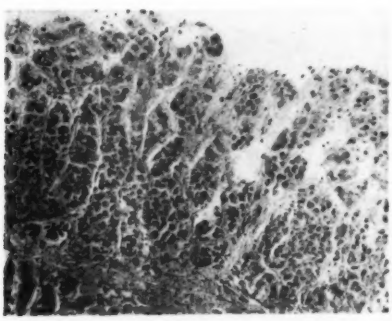

Fig. 10. Adrenal gland of the specimen after 85,000 units of Carzinophilin (Case No. 12).
Dissociation and coagulative necrosis of the cortex prominent.

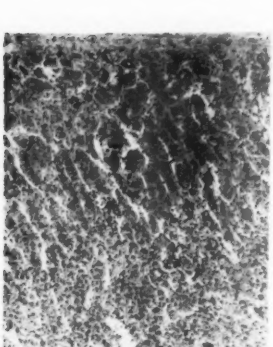

Fig. 11. Adrenal gland of the specimen after 78,000 units of Carzinophilin and 220 units of ACTH (Case No. 11). The cortex is kept comparatively intact and the cells are solidly filled with cytoplasmic granules.

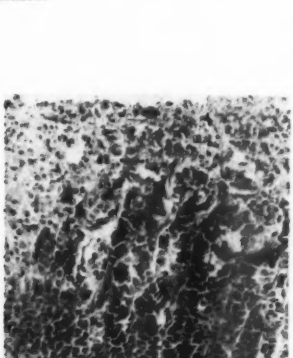

Fig. 12. Adrenal gland of the specimen in Fig. 3.
Pseduo-cavity formation and differentiation of the light and dark cells are noted in the zone glomerulosa adult and the cells in the fascicular zone show focal atrophy and dissociation.

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Demonstration of Defect of Creatinephosphokinase in Muscle of Progressive Muscular Dystrophy

By

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Although a disturbance in creatine metabolism and/or in carbohydrate metabolism has been presumed in progressive muscular dystrophy, it is uncertain where in metabolism of the muscle a primary lesion exsits.

This paper reveals that a primary lesion in the disease lies in a defect of creatinephosphokinase in the muscle as the results of enzymological examinations of sera, erythrocytes and muscles from two patients with progressive muscular dystrophy.

METHODS AND MATERIALS

Two cases of typical progressive muscular dystrophy with pseudohypertrophy (Case 1, 10-year-old boy; Case 2, 7-year-old boy) were subjected to this investigation.

Enzyme activity in serum

Aldolase was determined by Sibley's method.[1] Activities were expressed as the number of c.mm of fructose-1,6-diphosphate split per ml of serum per hour at 38°C.

Lactic dehydrogenase (LD) was determined by Hill's method.[2] Activities were expressed as one unit for a decrease of optical density of 0.001 at 340 mμ per minute per ml of serum.

Transaminases (GPT and GOP) were determined by Sigam-Frankel's method.[3]

Enzyme activity in erythrocytes

Aldolase and LD were determined by the above-mentioned methods. Activities were expressed per ml of erythrocytes.

Glucose-6-phosphate dehydrogenase (G6PD) was determined by Zinkham's method[4]. The activities[5] were expressed as one unit for an increase of optical density of 0.001 at 340 mμ for 15 minutes at 37°C, using 0.1 ml of 5% hemolysate of erythrocytes.

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The ATP content in blood was determined by Goto's method,[6] as "pyrophosphate" fraction obtained by ten minutes' hydrolysis with boiling normal hydrochloric acid, after which the liberated inorganic phosphorus was measured by Lohmann's procedure. The content was expressed as mg of P per 100 ml of blood.

The pyridine nucleotide (PN)—DPN and TPN—content was determined by Huff's method.[7] The content was expressed as γ per ml of erythrocytes.

Glucose uptake test by erythrocytes

Erythrocytes separated from heparinized blood were three times washed with physiologic saline after the removal of buffy coat. The mixture, consisting of erythrocytes 1 ml, plasma 0.5 ml, and isotonic glucose-saline phosphate buffer (pH 7.3, concentration of glucose 300 mg%, water-soluble penicillin 500 units per ml) 0.5 ml, was shaken at 37°C. At intervals of 0, one and two hours during the incubation, the concentration of glucose in the mixture was determined in order to know the rate of utilization of glucose by erythrocytes.

Muscle biopsy

Muscle specimens, weighing about 3 g, were removed surgically from the gastrocnemius with pseudohypertrophy of each patient. As controls, orthopedic patients without muscular disorder were subjected to muscle biopsy. The removed muscle specimens were washed with isotonic KCl solution and weighed, and immediately homogenized with a chilled 0.05 M KCl solution under cold condition. The homogenates, thus prepared at 0°C, were analysed according to the following procedures:— Micro-Kjeldahl's method for total nitrogen; Folin's method[8] for creatine and creatinine; Kahan's method[9] for glycogen; Eggleton's method[10] for ATP and creatine phosphate, and these contents were expressed as mg of phosphorus. Aldolase, transaminase (GOT) and LD were determined as described above.

Creatinephosphokinase: In order to exclude the interference by ATPase and myokinase both of which were abundantly contained in the muscle, creatinephosphokinase was isolated from the homogenate according to Lardy's method.[11] Then the isolated enzyme fraction was lyophilized and was dissolved in 0.1 M glycine buffer (pH 9.0) for assay of the enzyme activity. The reaction mixture consisted of the following: 0.080 M creatine, 3.0 ml; 0.100 M $MgSO_4$, 0.6 ml; 0.005 M ATP (Na^+), 2.0 ml; 0.1 M glycine buffer (pH 9.0), 2.4 ml; the enzyme solution to be tested, 2.0 ml. The mixture was shaken at 30°C for 30 minutes. At the end of the incubation, 2.0 ml of the mixture was pipetted into calibrated Evelyn tubes containing 1.0 ml of 5 per cent $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.8 ml of 60 per cent $HClO_4$ and distilled water to bring the total volume to 9.6 ml. After standing exactly 30 minutes at room temperature (about 23°C), 0.4 ml of 1,2,4-aminonaphthol sulfonic acid solution was added. After exactly 10 minutes for color development, the inorganic phosphate liberated from creatine

phosphate was determined at 660 mμ. The reaction mixture, in which H_2O replaced creatine, was employed as a control. The activities were expressed by difference of inorganic phosphate between the both mixtures.

Urinary excretion of creatine and creatinine was determined by Folin's method.[8]

RESULTS

As was shown in Table I, an increase of creatine and a decrease of creatinine in urine, thus a remarkable elevation of creatine-creatinine ratio were observed in the cases of progressive muscular dystrophy.

Table I. Urinary Excretion of Creatine & Creatinine.

		Progressive muscular dystrophy	
		Case I	Case II
Creatine	mg/day	150	200
	mg/day, kg of body weight	6.25	10.0
Creatinine	mg/day	145	167
	mg/day, kg of body weight	6.04	8.35
Creatine / creatinine ratio		0.97	0.84

The activities of serum aldolase, transaminase and LD were elevated, especially those of the former two enzymes, as was shown in Table II.

The activities of enzymes in erythrocytes, including aldolase, LD and G6PD, were all within normal limits. The contents of ATP and PN also showed no abnormal value (Table III). The glucose uptake test by erythrocytes resulted

Table II. Enzyme Activity in Serum.

	Normal range	Progressive muscular dystrophy	
		Case I	Case II
Aldolase (units/ml)	3~10	42	80
Transaminase (units/ml)			
GPT	12~24	120	122
GOT	12~24	112	148
Lactic dehydrogenase (units/ml)	50~300	380	450

within a normal range (Fig. 3).

Analytical results of the muscle specimens are shown in Table IV. The values per 1 g of muscles, for chemical components and enzymes tested, were all

TABLE III. Enzyme Activity in Erythrocytes.

	Normal range	Progressive muscular dystrophy	
		Case I	Case II
Aldolase (units/ml)	700~900	840	852
Lactic dehydrogenase (units/ml)	2×10^4~4×10^4	2.5×10^4	3.8×10^4
Glucose-6-phosphate dehydrogenase (units)	150~200	155	180
ATP (mg%)	3.5~4.5	3.8	4.0
Pyridine nucleotide (DPN & TPN) (γ/ml)	90~125	112	120

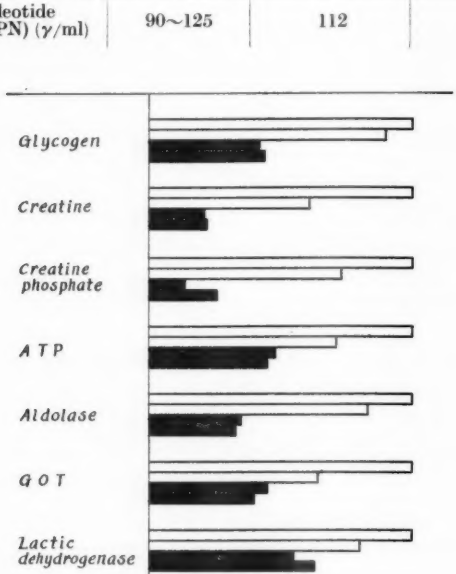


Fig. 1. Analysis of muscle in progressive muscular dystrophy (1)

lower as compared with controls (Fig. 2). When the values were calculated in terms per 1.0 g of nitrogen in the muscle, namely, on protein basis, the contents of glycogen and ATP and the activities of aldolase, transaminase and LD did not much differ from those of controls. In contrast to those, the activity of creatinephosphokinase was markedly reduced and the contents of creatine, creatinine

TABLE IV. Analysis of Muscle in Progressive Muscular Dystrophy.

		Nitrogen (mg)	Glycogen (mg)	Creatine (mg)	Creatinine (mg)	Creatine phosphate (mg)	ATP (mg)	Aldolase (units)	GOT (units)	Lactic dehydrogenase (units)	Creatine-Phosphokinase (units)
Progressive muscular dystrophy	Case I	9.25	1.08	0.672	unmeasuable*	0.08	0.15	5900	17600	83×10^3	110
			(117)	(72.6)		(8.65)	(16.2)	(639×10^3)	(1900×10^3)	(8960×10^3)	(119×10^2)
	Case II	12.3	1.12	0.695	unmeasuable	0.15	0.14	5500	15350	94×10^3	220
			(96.5)	(60.0)		(12.9)	(11.4)	(475×10^3)	(1325×10^3)	(8100×10^3)	(190×10^2)
Control	I	22.8	2.58	1.88	0.0321	0.58	0.31	17000	25000	150×10^3	820
			(117)	(85.4)	(1.46)	(26.4)	(14.1)	(773×10^3)	(1135×10^3)	(6820×10^3)	(373×10^2)
	II	21.5	2.31	3.085	0.0079	0.42	0.22	14100	389000	120×10^3	1158
			(107)	(144.0)	(0.37)	(19.5)	(10.2)	(650×10^3)	(1810×10^3)	(5600×10^3)	(538×10^2)

Results are given as value per 1 g of muscle, ( ) shows value per 1 g of nitrogen in muscle.

* "unmeasurable" means below 0.0050 mg/g.

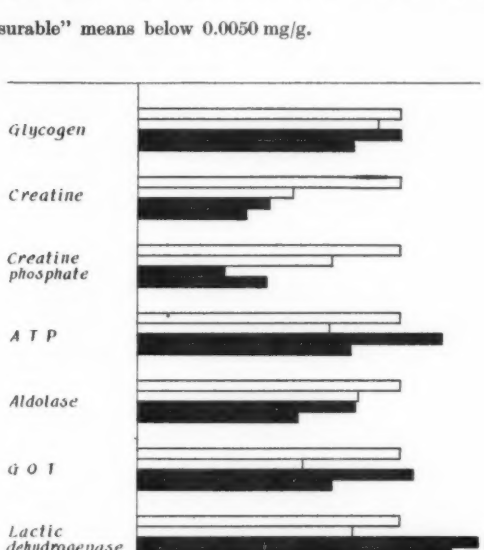


Fig. 2. Analysis of muscle in progressive muscular dystrophy (2)

and creatine phosphate were definitely lower as compared with those of controls (Fig. 3). Pathological findings of the biopsied muscles are shown in Fig 4.

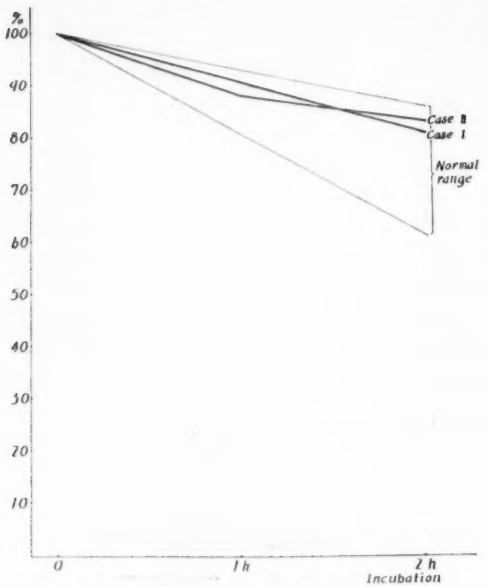


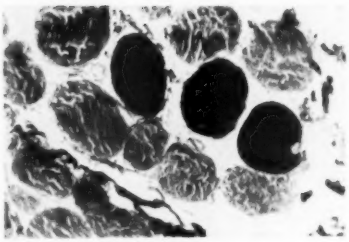
Fig. 3. Glucose uptake test by erythrocytes

There was partially evidence of hyaline degeneration, accompanied with an increase of adipose tissue and a proliferation of connective tissue. On the whole, a relatively early stage of pictures of progressive muscular dystrophy was observed.

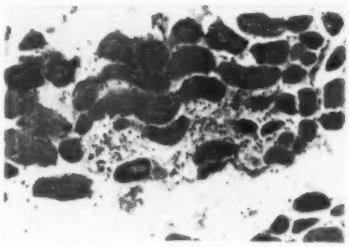
DISCUSSION

Since progressive muscular dystrophy is characterized by an increase of creatine and a decrease of creatinine, thus a remarkable elevation of creatine-creatinine ratio in urine, the elucidation of creatinuria may be a key to the mystery of the disturbance in metabolism of the muscle in the disease. The cause of creatinuria in the disease, however, has yet been inconclusive. Hoagland[12] described that creatinuria in progressive muscular dystrophy might be due to an unbalance between synthesis of creatinine in the liver and its utilization in the muscle. An abnormality of phosphagen synthesis due to the disturbance in crabohydrate metabolism in the muscle as reported by Meldoles[13),14] and a defect of phosphagen synthesis in the muscle as reported by Collazo[15] were presumed. No direct proof of where in the metabolism of muscle a block exists has been obtained probably due to a still insufficient enzymological study.

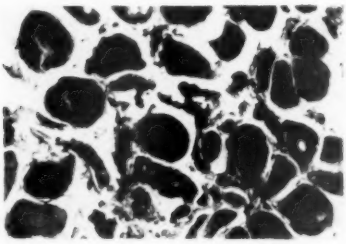
The evidence[15–17] that the content of phosphagen (creatine phosphate)



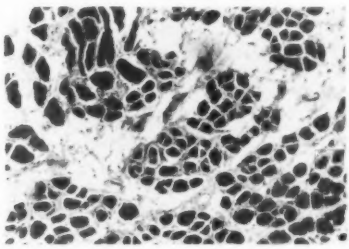
(a) Case 1. Mallory's stain



(c) Case 1. Hematoxylin-eosin stain



(b) Case 2. Mallory's stain
(a) and (b): Hyaline degeneration



(d) Case 2. Hematoxylin-eosin stain
(c)and (d): An increase of adipose tissue and a proliferation of connective tissue

Fig. 4. Pathological findings of the muscle of progressive muscular dystrophy

diminished in the muscle of progressive muscular dystrophy may indicate a disturbance in phosphorylation of creatine in the muscle of progressive muscular dystrophy. The phosphorylation of creatine in the muscle is catalyzed by creatinephosphokinase: Creatine + ATP⇄Creatinephosphate + ADP. The disturbance in this process, therefore, may result from one or more of the following possible items; 1) a decrease of creatinephosphokinase activity, 2) a decrease of ATP regeneration (energy production) due to a disturbance somewhere in glycolysis system, or 3) a decrease of creatine synthesis in the liver.

Since there are reports[12] that the synthesis of creatine in the liver may be normal in the disease and that creatine is excreted in excess in urine, the third possibility may be excluded. For the remaining two possibilities, a conclusion may be brought from the results obtained in this paper.

Studies on enzyme activity in serum in progressive muscular dystrophy demonstrated an elevation of activities of aldolase,[18-27] LD,[24] phosphoglucomutase,[28] transaminase,[29] and creatinephosphokinase.[30] Our results in this paper also indicated an elevation of activities of aldolase, LD and transaminase, especially 4 to 8 times higher activity of aldolase and 5 times higher activity of transaminase as compared with normal level. Since these enzymes exist

abundantly in the muscle, the elevation of their activities in serum is thought to be caused by leak of the enzymes from the degenerated muscle into the serum. The activities of aldolase and transaminase in serum may increase by destruction of even a small amount of muscle, because the activities of these enzymes are over 100 times higher in the muscle than in serum.[1),31),32)] Therefore, an elevation of activities of these enzymes in serum will not necessarily mean an abnormality of their activities in the muscle, although it has a meaning for evaluating the severity of degeneration of muscle.

On the assumptions that a certain disturbance in carbohydrate metabolism would exist in the disease and that such a disturbance would be detected in the erythrocytes, the activities of enzymes involved in glycolysis were investigated on erythrocytes of our own patients. The results obtained were all within normal limits (cf. Table III). In addition, the glucose uptake test by erythrocytes from the patients revealed normal figures (cf. Fig. 1). These results might suggest that there is no disturbance in carbohydrate metabolism in erythrocytes of progressive muscular dystrophy.

Nevin[16)] and Reinhold[17)] reported the decrease of creatine, creatinephosphate and ATP in the muscle of progressive musclar dystrophy as compared with those in the normal muscle. According to Ronzoni,[33)] both creatine and creatine phosphate were decreased while ATP was of normal content in the muscle of the disease. Dreyfus[34)] described lower activities of aldolase, phosphorylase and phosphoglucomutase in the muscle of the disease. Our results of muscular analysis revealed all lower values for the chemical components and the enzyme activities tested, when the values were expressed as those per weight of muscle (cf. Table III and Fig. 2). This is to be expected since the affected muscle tissues are in part degenerated, replaced by adipose tissue and connective tissue, as is shown in Fig. 4. Accordingly, when the values were calculated in terms of protein basis, there was not a great difference in the contents of glycogen and ATP and in the activities of aldolase, transaminase and LD between the affected muscle and the control. In contrast to that, the activity of creatinephosphokinase and the contents of creatine, creatinine and creatine phosphate were definitely decreased in the affected muscle as compared with those in the controls (cf. Fig. 3).

Basing upon the above-mentioned results, the following conclusions were drawn. The intact part of the muscles may maintain considerably normal activity in the glycolysis system and there may be no primary lesion in ATP regeneration through this system. On the other hand, the activity of creatinephosphokinase is decreased even in the intact part of the muscles and the deficit of the enzyme may be a defect in the metabolism of the muscle in progressive muscular dystrophy. A block in phosphorylation of creatine due to a defect of creatinephosphokinase may result in a decrease of creatine phosphate which

plays an important part as a high energy reserve in the muscle, and finally result in the decrease of muscular power. The fact that the lesion in progressive muscular dystrophy is confined to the muscle could be understood from the view that the defect of the enzyme is a primary cause in the disease, since creatinephosphokinase exists only in the muscle throughout the body. The creatinuria, a characteristic of the disease, also may be interpreted as follows. Creatine, one of amino acids, is throught to be easily permeable to cell membrane, but creatine phosphate may not be easily permeable and may be fixed in the muscle. In progressive muscular dystrophy, creatine will be excreted in excess in urine without being fixed in the muscle because of diminished phosphorylation. It is quite appropriate that Tripoli and Beard[35] used the name "Diabetes creatinurics" for the disease.

It may be worthy of notice that the two cases investigated in this paper were at a relatively early stage of the disease as is shown in the pathological findings of the muscle (cf. Fig. 4). In a much later stage of the disease, the carbohydrate metabolism may not be intact because both metabolisms of creatine and of carbohydrate have relation to each other in the muscle.

SUMMARY

Enzymological studies on serum, erythrocytes and muscle were carried out in two patients with progressive muscular dystrophy.

The activities of serum aldolase, transaminase and LD were remarkably elevated.

The activities of the erythrocyte enzymes such as aldolase, LD and G6PD were within normal limits. The contents of ATP and PN in erythrocytes showed no abnormal values. The glucose uptake test by the erythrocytes revealed normal findings.

Analytical results of the muscle revealed that the activity of creatinephosphokinase and the contents of creatine, creatinine and creatine phosphate were definitely lower, while the content of glycogen and ATP and the activities of aldolase, transaminase and LD did not differ much from those of controls in the values per 1.0 g of nitrogen in the muscle.

CONCLUSIONS

A disturbance in phosphorylation of creatine due to a defect of creatinephosphokinase is a site of block in the metabolism of muscle in progressive muscular dystrophy, while the intact part of the muscle may maintain considerably normal activity in glycolysis and there may be no primary lesion in ATP regeneration through the glycolysis system.

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